Immunochemistry of the acetylcholine receptor.

Bird, Martin Charles

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IMMUNOCHEMISTRY OF THE ACETYLCHOLINE RECEPTOR

Submitted by MARTIN CHARLES BIRD

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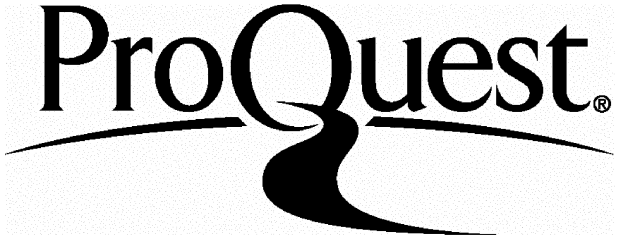

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CONTENTS

SUMMARY

1. Anti-Torpedo marmorata AChR antibody fragments (Fab and $F(ab')_2$) have been prepared from sheep immunised with Torpedo AChR, and exhibiting experimental autoimmune myasthenia gravis. The antibody fragments were labelled with ^{125}I with the retention of antigen binding capacity. Labelled and unlabelled antibody fragments were used to study the antigenicity of soluble and membrane-bound AChR. Anti-receptor antibodies were found to reduce α-toxin binding to the AChR. This was demonstrated to be caused by steric hindrance rather than by direct blockade of the toxin binding site, or by antigenic modulation of the receptor. Removal of carbohydrate residues from the AChR resulted in no decrease in antibody binding, implying that carbohydrate made no direct contribution to the antigenicity of the receptor. Denaturation of the AChR resulted in a decrease in anti-receptor antibody binding of between 60 and 84%. Thus antibodies were directed mainly at conformation-dependent sites on the receptor. Substantial differences between the antigenic sites of soluble and membrane-bound receptors were found.

2. The contribution of antibody-mediated muscle cell lysis in the pathogenesis of myasthenia gravis has been studied, using a novel *in vitro* cytotoxicity assay based on ^{3}H-carnitine uptake and release by muscle cells in culture. Cytolytic activity toward both chick and human embryonic muscle cell cultures was demonstrated in over 30% of myasthenic sera, suggesting that myolysis may be a major mechanism for AChR loss in myasthenia gravis. Heat-inactivation of the sera abolished their lytic activity, and it was not fully restored by the addition of guinea pig complement. Myolysis may be caused by antibodies other than anti-AChR antibodies present in myasthenic sera.

INTRODUCTION

Historical

The understanding that the function of nerves is to carry messages dates back to early Greek physicians. Erasistratus of Chios (circa 310-250B.C.) believed that "animal spirits" flowed from the ventricles of the brain down the hollow nerves to make the muscles swell, and thus to shorten. This concept occurred throughout the history of investigation of peripheral nerve function until as late as the 17th century, when Jan Swammerdam (1637-80) the Dutch embryologist and microscopist introduced the nerve-muscle preparation, and showed that crushing a nerve induced contraction of the muscle without a change in muscle volume.

Until the work of Ramón y Cajal (1852-1934) in 1888 the nervous system was regarded as a syncytium or nerve net, and thus a continuous network. In 1839 Theodore Schwann had enunciated the Cell Theory, stating that the body was composed of individual units or cells, and Cajal, from studies of metal-stained preparations of the nervous system, demonstrated the presence of separate cells in this system also. In 1891 von Waldeyer summarized all the evidence, starting from Schwann's Cell Theory, and named the basic unit or cell of the central nervous system the "neuron". Among Cajal's discoveries was that of the "bouton terminaux" by which neurons communicated with other cells. This anatomical finding was later developed by Sir Charles Scott Sherrington, who in 1906 published "The Integrative Action of the Nervous System", in which he introduced the term "synapse" to describe Cajal's bouton terminaux.

Claude Bernard in 1856 showed that injection of curare produced paralysis in frog leg muscle and also that the curare-

poisoned leg muscle could not be stimulated electrically via its nerve but was nevertheless fully sensitive to direct electrical stimulation. These classic experiments were clearly the forerunners for the investigation of synaptic transmission, neurotransmitters, and indeed the whole field of neuropharmacology. However, it was only following the realisation that the nervous system was electrically discontinuous that the presence of some additional form of communication across the synapse became of paramount importance.

The beginning of the 20th century saw the elucidation of the pharmacology of the autonomic nervous system, which had an important bearing upon the understanding of all synaptic events. In 1904 Thomas Elliott discovered that adrenaline had an effect similar to stimulation of the sympathetic nervous system, and suggested that adrenaline was released by sympathetic nerve activity. In 1906 W.E. Dixon similarly deduced that muscarine or a muscarine-like compound was stored in parasympathetic nerve endings. These two discoveries formed the basis of the idea of neurohumoral transmission, in which a chemical messenger is released at the synapse, and conveys the message to adjacent cells. In 1921 Otto Loewi identified a chemical, called by him "Vagustuff" which was released from the stimulated vagus nerve. In 1914 Sir Henry Dale had discovered acetylcholine in extracts of ergot, and later showed that Loewi's vagustuff was, in fact, acetylcholine. Eventually,in 1936 Dale and his colleagues Vogt and Feldberg demonstrated that acetylcholine appeared after direct nerve stimulation, and that it could activate striated muscle. Thus the chemical theory of synaptic transmission was finally born.

It is now recognised that several criteria must be satisfied

before a chemical mediator can be established as a neurotransmitter (Werman, 1966). These include the following:

1. The mediator is synthesized and stored in the nerve terminal.
2. The mediator is released on nerve stimulation, and this release must be calcium-dependent.
3. The mediator exerts its effect at a post-synaptic site by inducing a change in polarisation of the post-synaptic cell.
4. There exists a method for removal of the mediator.

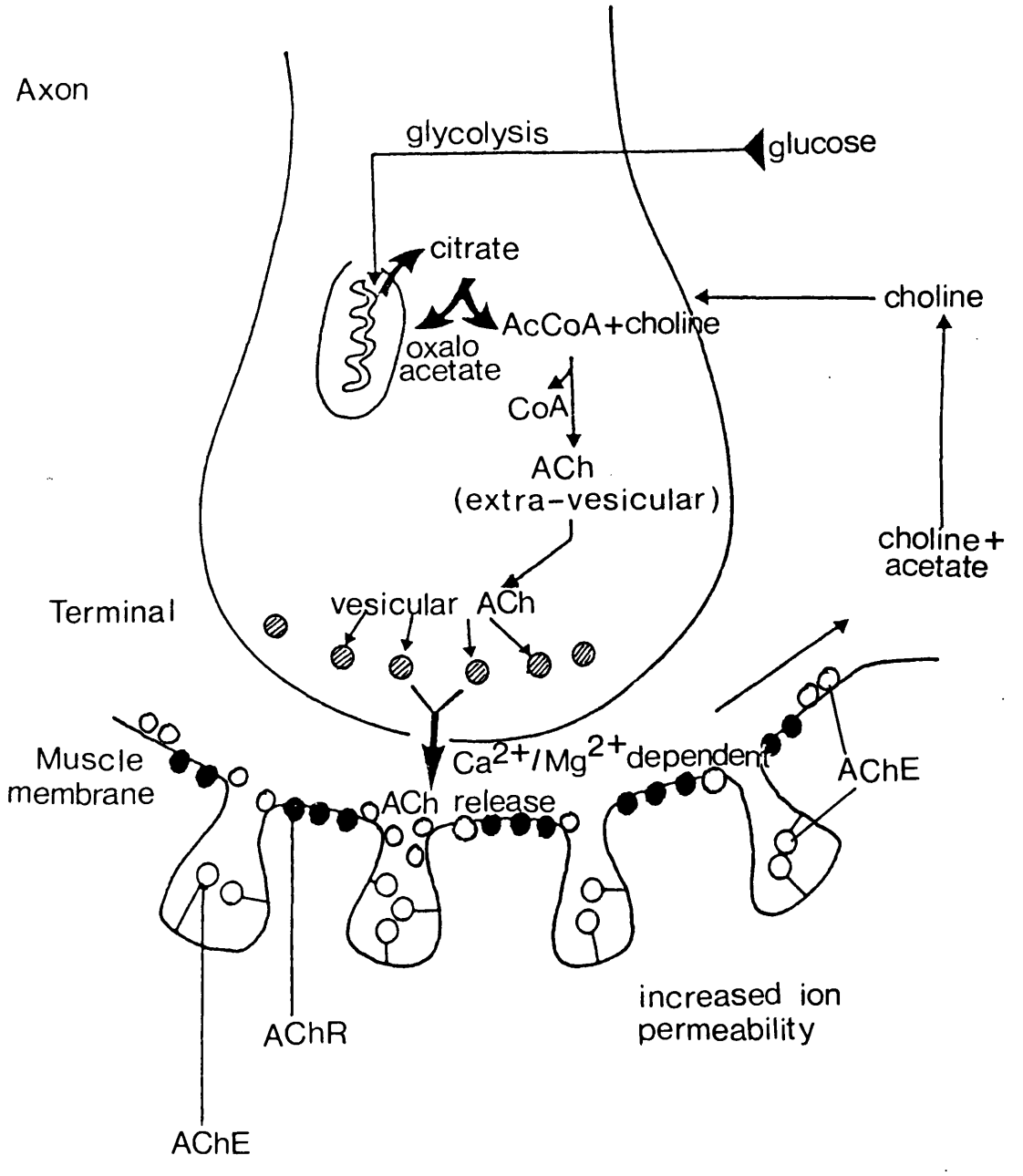
The neuromuscular junction and synaptic transmission

The best elucidated system of synaptic transmission is the neuromuscular junction. Its function is to transfer the propagated electrical impulse from the α-motoneuron to the muscle, leading ultimately to muscle contraction. It is now well established that acetylcholine is the only neuroactive agent at the vertebrate motor-end plate, and it has been shown that it satisfies all the criteria for a neurotransmitter.

The anatomy and physiology of the vertebrate neuromuscular junction have been extensively studied, not only because of its influence on muscle action, but also because of its ready accessability as a peripheral synapse, and the significant information that it contributes to an understanding of synaptic morphology and function. The basic features of the cholinergic terminal at the vertebrate neuromuscular junction are shown schematically in Figure 1.

As the motor axon approaches the muscle fibre it loses its

FIGURE 1. The organisation of the cholinergic terminal at the vertebrate neuromuscular junction.

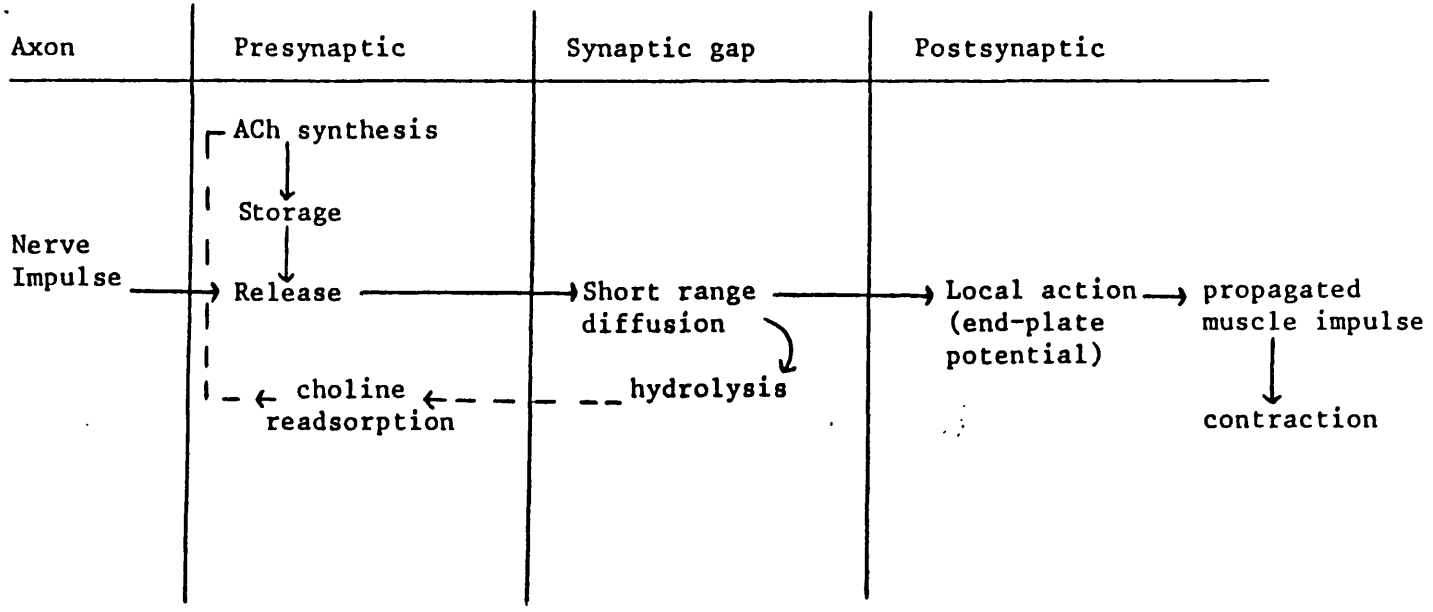


myelin sheath and divides into several small branches. These branches lie in shallow grooves on the surface of the muscle fibre and run short distances before terminating. The region of the muscle fibre under the branches is termed the motor-end plate; it includes the sarcolemma and also a mound of sarcoplasm, the sole-plate. Within the sole-plate are collected a number of muscle fibre nuclei as well as many mitochondria, ribosomes and pinocytic vesicles. Lying over the whole end-plate are membrane-covered cytoplasmic processes derived from the Schwann cells. With the electron microscope it can be seen that the membrane of the axon terminal is separated from the sarcolemma by a distinct gap measuring about 50nm - the primary synaptic cleft. The primary cleft is interrupted by repeated invaginations of the sarcolemma into the sole-plate. Each fold forms one secondary synaptic cleft. The secondary clefts are approximately 0.5 to 1μm deep and are wider (70-90nm) at their terminations than at their necks (50nm). The electron microscope also shows that the basement membrane on the outside of the sarcolemma runs through both primary and secondary clefts to emerge on the far side of the junction. Like the muscle fibre, the axon is also highly specialised at the neuromuscular junction. The axon terminations contain large numbers of small spheres with membranous linings. They measure some 30nm across and are typically clustered opposite the secondary synaptic clefts. These spheres are the synaptic vesicles, and contain acetylcholine. It has been suggested that the vesicles are formed by the pinching off of neurotubules or endoplasmic reticulum within the axon terminal (Blumcke and Niedorf, 1965). The axoplasm also contains two other types of vesicle, though in much smaller numbers. The dense-cored vesicles (70-110nm) resemble those known to contain

monoamine transmitters in the central nervous system, and it is conceivable that their contents are similar at the neuromuscular junction. An alternative suggestion is that they contain neurotrophic messenger molecules. The coated vesicles apparently form by in-pouching of the axolemma, and presumably enable the axon terminal to take up impermeant molecules from the synaptic cleft. Also within the axon terminal are large numbers of mitochondria which probably provide most of the energy, in the form of ATP, required for the synthesis of acetylcholine. Finally the axon terminal contains large numbers of neurotubules and neurofilaments which enter from the axon fibre.

A relatively satisfying account of the synaptic events which enable excitation to spread from the motor axon to the muscle fibre is possible, not only because many details of the transmission process are understood, but also because many structural features of the synapse can be correlated with function. The process of synaptic transmission is shown schematically in Figure 2. The first step in transmission is the propagation of the action potential into the distal regions of the motor axon. The impulse travels all the way to the synapse itself, as shown by Katz and Miledi (1965) who were able to record a propagated potential with an extracellular microelectrode positioned close to the axon terminal. When the impulse invades the axon terminal it depolarises the axon membrane. During this depolarisation calcium ions enter the axon from the extracellular fluid. Calcium ions are essential for the release of acetylcholine. It is possible that they activate sites on the inside of the axolemma, so allowing fusion to take place between this membrane and that of the synaptic vesicles. The vesicles then discharge their acetylcholine into the synaptic cleft. Most of the synaptic delay

FIGURE 2. Schematic representation of the organisation of the vertebrate neuromuscular junction. (After Katz,1966)



between the excitation in the nerve, and subsequently in the muscle fibre can be accounted for by the calcium-mediated release of transmitter. In mammalian junctions this delay is of the order of 0.2 milliseconds. Once the acetylcholine is discharged into the cleft it diffuses very quickly towards the muscle fibre membrane. The fall in muscle fibre membrane potential following an impulse in the motor-axon is termed an end-plate potential (E.P.P.). The E.P.P. results from an increase in the permeability of the membrane to both sodium and potassium ions initiated by the interaction of acetylcholine with the membrane. Under normal circumstances the developing E.P.P. is overtaken by an action potential, which arises as soon as the critical membrane potential has been exceeded (Fatt and Katz, 1951). The impulse is set up in the membrane at, and immediately adjacent to, the synapse, and is then propagated in both directions along the fibre. The sequence of events in the transmission process is concluded by the hydrolysis of acetylcholine, to choline and acetate, catalysed by the enzyme acetylcholinesterase.

The acetylcholine receptor (AChR)

The nature of the interaction of acetylcholine with the muscle membrane has only been elucidated fully comparatively recently. The first experimental evidence of the possible existence of a receptive substance for acetylcholine at the neuromuscular junction was, however, provided by Langley as early as 1907. Direct evidence that the cholinergic receptors were localised at the cell membrane, and that they were orientated toward the extracellular space was reported in 1955 with the introduction of the iontophoretic application of acetylcholine at the myoneural junction. The elegant experiments of del Castillo and Katz demonstrated that the receptor could be

activated only when the transmitter was applied extracellularly and not when it was injected inside the cell. These findings thus localised the search for an AChR to chemosensitive sites on the post-synaptic membrane at the synaptic junction.

The first report of purification of an AChR was made by Chagas and Ehrenpreis (reviewed by Hasson-Voloch, 1968). The pharmacology of this preparation was, however, suspect. In 1967 De Robertis, Fiszer and Soto used a similar approach to isolate a receptor protein from rat brain which bound cholinergic ligands. The problem of such tissue, however, is that it contains a mixed population of different receptors. Thus, for the isolation and characterisation of the AChR to be feasible, a rich source of relatively pure receptor was necessary. A second problem was to find a specific receptor marker by which the progress of the AChR through any purification procedure could be monitored. The first of these problems found its solution in the electric organs of the electric fishes.

The electric organs of the electric fish

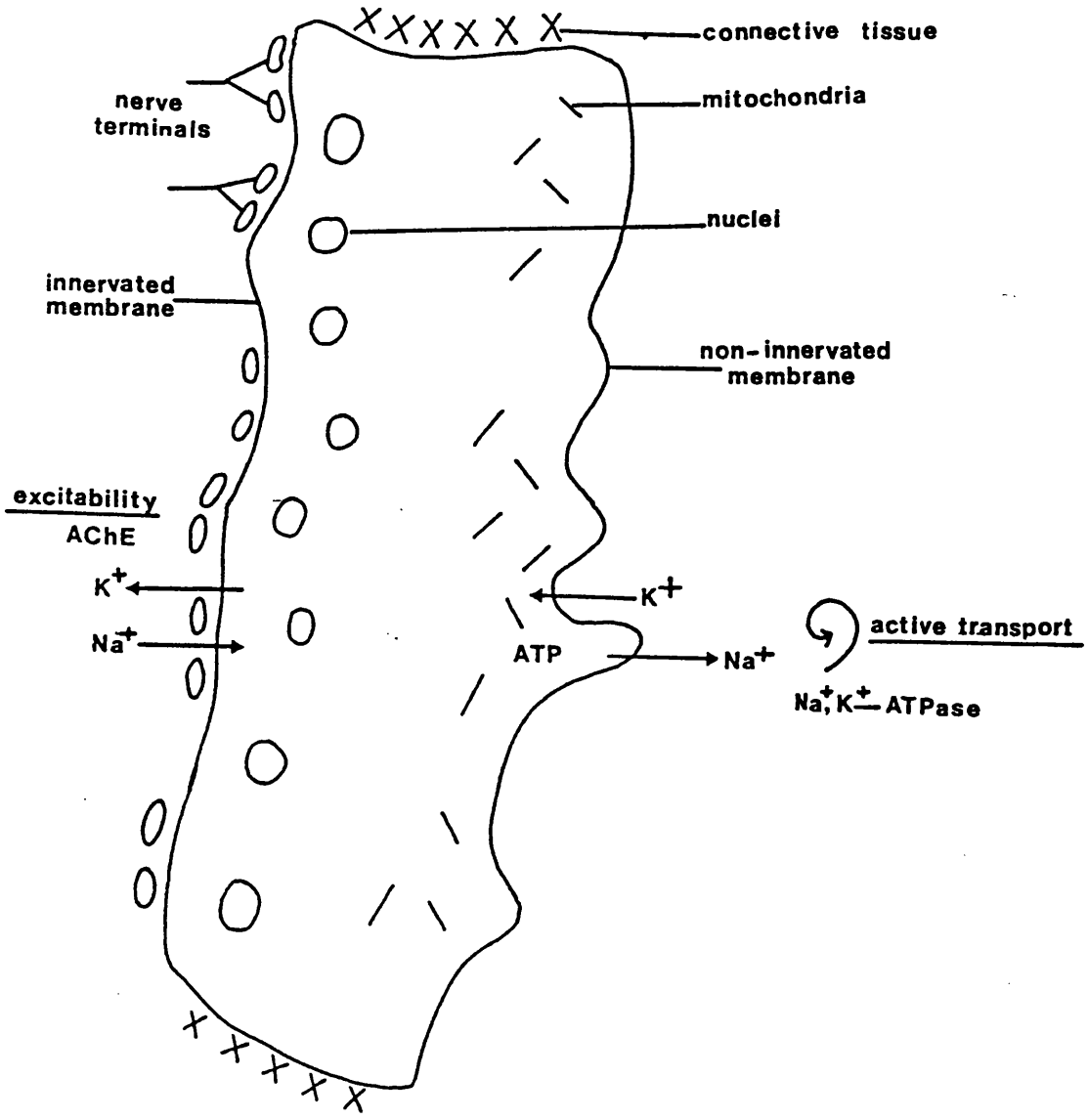
The electric organs of electric fish consist of arrays of large flat cells called electroplaques, each of which is capable of producing a change in potential on receiving a nerve impulse. The potential generated by each electroplaque is of the order of 0.14 volts. As the cells are arranged in series, the discharge produced is the summation of the evoked potentials. The two most studied fish, *Electrophorus electricus* (the electric eel) and *Torpedo marmorata* (electric ray) can generate potentials of 600 and 40-60 volts respectively.

The electroplaque of both species is a giant syncytium containing several thousand nuclei. It is a highly assymetric cell, receiving nerve terminals on only one of its faces - the caudal face in Electrophorus, the ventral in Torpedo. The plasma membrane on both surfaces shows a remarkable increase of surface area as a consequence of the proliferation of invaginations and villosities. In Electrophorus, nerve endings establish 10^5 to 10^6 specific contacts with one electroplaque, but cover only 1.4 to 2% of the surface of the membrane on the innervated face. In Torpedo innervation is much denser, and the innervated membrane less convoluted, so that the subsynaptic areas occupy up to 50% of the total surface of the innervated membrane. Thus electric organs from Torpedo can be expected to be much richer in receptor than those of Electrophorus (Changeux, 1975). Figure 3 shows a schematic representation of an isolated electroplaque.

In 1877 Du Bois-Reymond realised that the electric discharge produced in the electric organs was generated in the same way as that at the neuromuscular junction. Embryologically the electric organ develops from the same tissue as skeletal muscle. Although, the contractile elements are absent from the electroplaques they nevertheless respond to acetylcholine in a similar way to muscle cells. In the electric organs of Electrophorus the response to acetylcholine is the generation of an action potential analogous to that seen in a muscle cell. The electroplaque of Torpedo, on the other hand, is unable to generate an action potential and the discharge of the cell derives from the summation of individual excitatory end-plate potentials. In both types of electroplaque the net influx of current passes through the non-innervated face of the cell to complete the circuit (Lester, 1977).

FIGURE 3. Schematic representation of an isolated electroplaque.

(After Changeux, 1975).



The electric organs of the electric fish are an ideal model for the study of neurotransmission at the neuromuscular junction, as they possess similar excitability properties. The organisation of the myoneural elements is such that all the mediators involved in the transduction process are present in abnormally high concentrations. This fact, together with the fact that the tissues are purely nicotinic cholinergic, rapidly made electric organs the tissue of choice for the isolation and purification of the AChR, and it is the extensive investigation of this receptor which has revealed most of the currently known characteristics of the AChR.

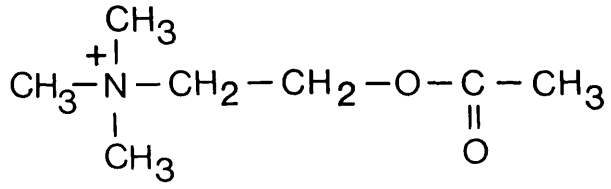
The pharmacological properties of the isolated electroplaque

Drugs which affect transmission are classified either as activators (agonists) if they cause depolarisation of the post-synaptic membrane, or as blockers (antagonists) if they block the depolarisation caused by acetylcholine or its agonists. A few drugs called "depolarising blockers" bridge the gap between the two main classes. Long before acetylcholine was recognised as a neuro-transmitter, neuromuscular junctions were classified into muscarinic and nicotinic types. The first of these are present in smooth muscle, and are activated by muscarine and inhibited by atropine. The second type is present in skeletal muscles, and is activated by nicotine, and inhibited by d-tubocurarine. Some examples of chemicals acting at the synapse are given in Figure 4.

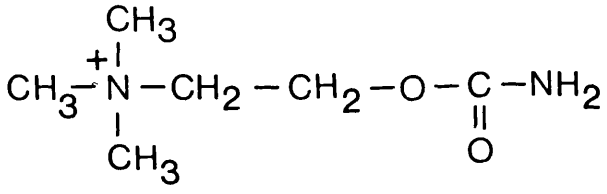
The pharmacological properties of the electroplaque have been well characterised (Nachmanson, 1959) and shown to be similar to those seen by Dale et al. (1936) at the neuromuscular junction. Agonist activity was found to be subject to strict structural limitations, requiring the presence of a trimethylammonium group

FIGURE 4. Cholinergic effectors.

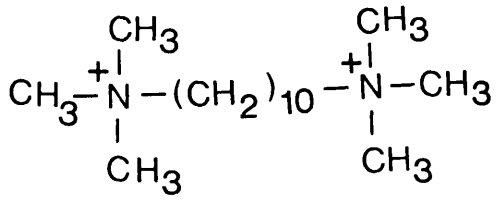
Agonists



Acetylcholine

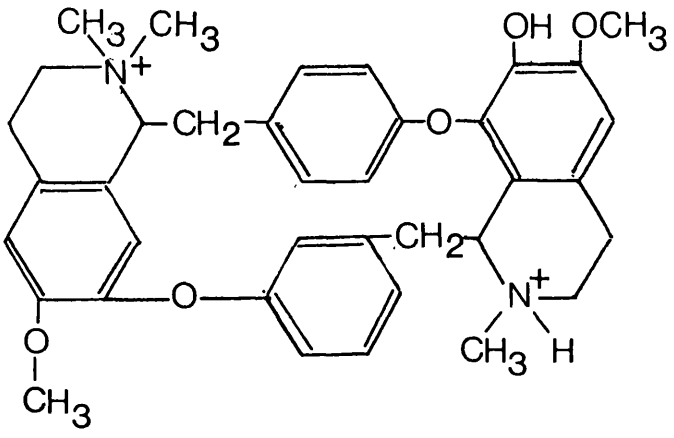


Carbamoylcholine

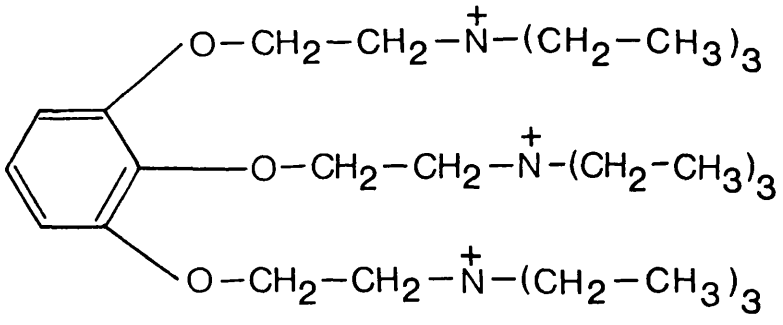


Decamethonium

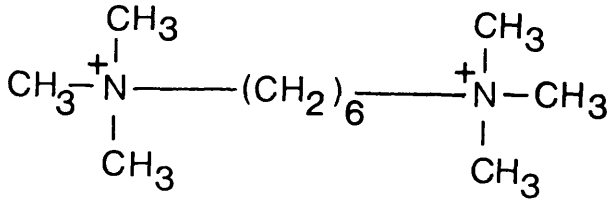
Antagonists



d-tubocurarine



Gallamine triethiodide



Hexamethonium

and a carbonyl oxygen at 0.59nm radial distance from a quaternary ammonium moiety. Concentration-effect curves for agonist response showed saturation, with a maximum response being observed in the presence of excess agonist. The shape of the curves was slightly sigmoidal, and determination of the gradient of Hill plots revealed cooperativity. The effect of an agonist was shown to reverse the blocking effect of an antagonist, and vice-versa, rather like the competitive interaction between substrate and inhibitor at an enzyme catalytic site. On this basis Nachmanson (1959) proposed that the receptor was a protein.

Evidence for the proteinaceous nature of the AChR was actually suggested as early as 1955, and fully established from about 1966 onwards. The work of De Robertis et al. (1967) and Silman and Karlin (1967) made it evident that the receptor protein was probably an intrinsic protein of the cell membrane, requiring quite drastic treatments for extraction from its lipid milieu. Changeux et al. (1969) isolated a membrane fragment from Electrophorus electricus that was rich in acetylcholine activity. In view of the suggestion that the AChR was an integral membrane protein Changeux et al. (1970) used the ionic detergent, sodium deoxycholate to solubilise a protein from the electric organ of Electrophorus. They showed agreement between the dissociation constants (K_D) for the binding of agonists and antagonists to the acetylcholinesterase-rich membrane fragments, the isolated electroplaque and the detergent-solubilised receptor.

Once a cholinergic tissue is homogenised the usual means of AChR identification in situ (the effect of drugs on the depolarisation of membranes or on muscle contraction) is lost. A

successful way to identify AChR in subcellular fractions is to study its binding to cholinergic ligands. However, binding of such ligands is not in itself sufficient for the identification of the receptor, because macromolecules other than AChR (e.g. acidic mucopolysaccharides or albumin) can act similarly. Thus a quantitative, reproducible assay for the detection of the isolated AChR must satisfy several strict criteria if the measurements obtained are to be a true representation of the ligand-receptor interaction in vivo. These criteria are outlined below.

I. The ligand used as a probe must be fully active biologically so that it can mimic the activity of the parent compound at the receptor site.
2. The binding observed must exhibit absolute structural and steric specificity, which must relate to the known biological activity of the parent ligand, its structural agonists and antagonists.
3. The binding should demonstrate saturability within a concentration range that can be meaningfully related to that of agonists which elicit the known biological response in intact biological systems.
4. The binding interactions should reflect an affinity in keeping with the sensitivity of the tissue to the physiologically active concentration of the ligand.
5. The presence of binding should be restricted to tissue known to be physiologically sensitive to the ligand.

Initially ^{14}C -decamethonium (Changeux et al., 197I) or ^{3}H -acetylcholine (Eldefrawi and Eldefrawi, 1973a) were used, with bound ligand being separated from free ligand by equilibrium

dialysis. However, a more sensitive and irreversibly-bound marker for the isolated AChR was necessary to assess accurately the purification procedure. The answer to this requirement came surprisingly through the serendipitous discovery of the mode of action of the elapid snake venoms.

The α-neurotoxins as probes for the AChR

Snakes from the elapid and hydrophobid families kill their prey by paralysing the respiratory muscles. In the late 1950's, Lee was able to explain the action of the venom in terms of its blockage of neuromuscular transmission (for review see Lee and Chang, 1966). Lee showed that very low concentrations of venom were capable of producing an anti-depolarising block at the neuromuscular junction very similar in action to that of acetylcholine antagonists. It did not affect the action potential of either the nerve or the muscle, the release of acetylcholine or its hydrolysis by acetylcholinesterase. The inhibitory action could be reversed by cholinergic effectors. Analysis of the crude venom showed that a single class of polypeptides, now called α-toxins, was responsible for the neuromuscular block by binding specifically to the AChR.

Two types of α-neurotoxin have been isolated. Type I produce a reversible neuromuscular blockade, whereas for type II toxins the blockade is essentially irreversible. Both types of α-toxins are compact, basic polypeptides. Type I toxins, (such as α-toxin from Naja naja siamensis) have 60-62 amino acid residues (Cooper and Reich, 1972), while type II (for example α-bungarotoxin from Bulgarus multicinctus) have 71-74 residues. α-Bungarotoxin (α-BGT) has a molecular weight of 8000, comprising 74 amino acid residues with 5 disulphide bridges, and an isoelectric point (pI) of 9.0 (Dolly, 1979).

Type II toxins are ideal probes for the AChR, and for this purpose it is possible to radiolabel the α-toxins with the retention of their biological activity. Several methods have been employed, including incorporation of ^{131}Iodine or ^{125}Iodine (^{125}I) into tyrosine residues (Miledi et al., 1971; Berg et al., 1972), partial methylation ($^{14}CH_3$) of amino groups (Biesecker, 1973) and tritiation by incorporation of an acetyl (Dolly and Barnard, 1974) or propionyl group (Barnard et al., 1979).

The radiolabelled α-toxins have proved invaluable as they can be used to identify the AChR both in solution, and in fragments of cell membrane, where electrophysiological measurements of receptor function are not possible. Radiolabelled α-BGT has now become almost universally used for the detection of AChR because of its great sensitivity, made possible by labelling to high specific activities, and because of the virtually irreversible nature of its binding (Dolly, 1979; Vincent and Newsom-Davis, 1979).

Isolation of electric fish AChR

Conventional procedures for the purification of the AChR, such as ammonium sulphate precipitation, DEAE - cellulose chromatography or hydroxylapatite chromatography, have generally not resulted in complete purification of the active AChR.

α-Neurotoxins of type I have proved to be ideal ligands for use in affinity chromatography of the AChR, and this technique has given a dramatic one-step purification of the receptor. α-Neurotoxins commonly used as immobilised ligands include those from N.naja (Karlsson et al., 1972; Lindstrom and Patrick, 1974) and Naja naja siamensis (Klett et al., 1973; Eldefrawi and Eldefrawi, 1973b).

Desorption of AChR from the affinity ligand is achieved by the use of cholinergic agents such as carbamylcholine, hexamethonium, or gallamine triethiodide.

Other ligands, such as synthetic quaternary ammonium derivatives, have also been successfully applied in the purification of AChR by affinity chromatography (for review see Fulpius, 1976).

The eluting ligand is easily removed by dialysis (Karlsson et al., 1972; Olsen et al., 1972), or by gel filtration (Shorr et al., 1978). Additional steps have been taken to purify the AChR to homogeneity. These include (1) ultracentrifugation in a sucrose density gradient (Lindstrom and Patrick, 1974), (2) chromatography on DEAE -cellulose (Klett et al., 1973) or DEAE -Sephadex (Dolly and Barnard, 1977), (3) preparative electrophoresis (Eldefrawi and Eldefrawi, 1973b) or (4) chromatography on lentil lectin-Sepharose resin (Shorr et al., 1978).

The receptor protein from electric fish has been purified to specific activities in the range of 2-12μmoles of α-BGT binding sites per gram protein (for review see Changeux, 1981) using the techniques outlined above. Protective measures to inhibit endogenous proteases must be taken in the isolation procedure in order for the native subunit structure of the AChR to be maintained (Lindstrom et al., 1980; see p.22 below).

Isolation of skeletal muscle AChR

Progress in the purification of the AChR from skeletal muscle has been much slower than that of electric fish because of the very low content of receptor in muscle sarcolemma (Wallis et al., 1980) and the susceptibility of the receptor to proteolysis during purification

(Lindstrom et al., 1980 ; Shorr et al., 1981; Einarson et al., 1982). Nevertheless, AChR purification has been reported from a number of vertebrate skeletal muscle sources (Table 1).

Properties of the isolated AChR

Molecular weight and molecular shape: Determination of the molecular weight of the purified receptor in solution can only give approximate values because of the presence of bound detergent. Meunier et al., (1972b) showed that Triton X100 can contribute up to 21% (160-170 molecules) of the total mass of the receptor-toxin complex in solution. Similarly Karlin et al., (1979) reported that the AChR binds 0.5g of Triton X100 per gram protein. Values for the molecular weight of all species range from 230000-370000 (for review see Changeux, 1981). Sedimentation of receptor or receptor-toxin complex in a sucrose density gradient showed that the protein sedimented with a sedimentation coefficient of $S_{20,w}$9S (Sobel et al., 1978; Biesecker, 1973; Dolly and Barnard, 1977; Froehner et al., 1977a). A second species in both electric organ of Torpedo californica (McNamee et al., 1975) and Torpedo marmorata (Barnard et al., 1978) has been found with sedimentation coefficient $S_{20,w}$13S, and has been shown to be a dimeric form of the receptor which is stabilised in the presence of N-ethyl maleimide (Barnard et al., 1978). Dimerisation is favoured at higher Triton X100 concentrations (Reinhard et al., 1981). The ratio of the Stokes radius to the minimum possible radius for the protein-detergent complex falls outside the range for globular proteins (Meunier et al., 1972b) and, indeed, neutron scattering measurements of the monomer and dimer in detergent solution are consistent with the molecule's being an oblate ellipsoid with an axial ratio of 4:1 (Karlin et al., 1979).

TABLE 1. The subunit composition of vertebrate skeletal muscle AChR.

SOURCE	SUBUNIT COMPOSITION (Daltons x 10^3)						REFERENCE
Cat denervated muscle	41						Shorr et al., 1978.
" " "	43						Shorr et al., 1981.
Rat denervated muscle		45	49	51	56	62	Froehner et al., 1977.
" " "	42					66	Kemp et al., 1980.
Rat innervated muscle		45	49	51	56	66	Nathanson and Hall, 1979.
Rabbit denervated muscle	42	44		52	58	68	Gotti et al., 1983.
Rabbit innervated muscle	42	44		52	58	68	" " "
Foetal calf muscle	42		49	55	58		Gotti et al., 1982.
" " "	42		49	55	58		Conti-Tronconi et al., 1982.
" " "	41		50	53	56		Lindstrom et al., 1979a.
Chick embryonic muscle	40		50	54			Sumikawa et al., 1982.
Innervated	40		50	54			" " "
Denervated	41		50	54			" " "
Adult human muscle	42				56	66	Stephenson et al., 1981.
" " "		44	53	56	61	66	Momoi and Lennon, 1982.

Subunit composition: The receptor has been shown to have an oligomeric structure; in the presence of SDS it splits into subunits of lower molecular weight (Karlin et al., 1971; Meunier et al., 1971, 1972a; Raftery et al., 1971). Analysis of the subunits by SDS polyacrylamide gel electrophoresis under denaturing conditions has shown that AChR from Torpedo marmorata and Torpedo californica has four subunits with molecular weights of 38-40000 (α), 50000 (β), 57000 (γ) and 64000 (δ) present in the ratio 2:1:1:1 (Barnard et al., 1979; Lindstrom et al., 1979a; Raftery et al., 1979).

In mammalian muscle the number of subunits has been variously reported at between one and five (Table 1). It now appears, however, that reports of less than four subunits are attributable to selective proteolysis of the higher molecular weight subunits (Lindstrom et al., 1980; Einarson et al., 1982).

The subunits of Torpedo AChR are acidic glycoproteins which can be distinguished by peptide mapping (Lindstrom et al., 1979b; Vandlen et al., 1979; Nathanson and Hall, 1979). Specific affinity ligands can also be used to label individual subunits (Karlin, 1974; Lyddiat et al., 1979; Karlin, 1980; Saitoh et al., 1980). The arrangement of subunits in the monomeric receptor is not known, but the subunits are known to be strongly noncovalently associated. Even after extensive proteolysis the proteolytic fragments remain associated, and both the ligand binding site and ion-channel of the receptor remain functional (Lindstrom et al., 1980; Conti-Tronconi et al., 1982).

Purified receptor is consistently associated with two additional protein components of M_r 43000 and 90-150000. The higher molecular weight compound is generally considered to be a component

of the Na^+,K^+-ATPase. Most recent evidence suggests that the 43000 protein is a peripheral protein associated with the cytoplasmic face of the membrane in situ, and possibly has a structural role (Barrantes et al., 1980; Lo et al., 1980; Wennogle and Changeux, 1980; Gordon et al., 1983).

Reconstitution of AChR in model membranes

Re-assembly of the detergent-solubilised, purified receptor into a membrane environment constitutes proof that the purified protein contains all the essential elements of the functional AChR. The AChR has now been reconstituted into lipid bilayer vesicles and planar lipid films with complete recovery of ligand binding and ion permeability properties (Anholt, 1981; McNamee and Ochoa, 1982).

Biochemical properties of the isolated AChR

In 1955 Nachmanson suggested that the AChR was a protein on the assumption that only proteins had the ability to "recognise" specific molecules. Amino acid analysis of the purified receptor confirms the protein nature of the AChR. The amino acid composition of the AChR is very similar to that of acetylcholinesterase, a finding which led to early suggestions that the two were identical (O'Brien et al., 1972). Devillers-Thiery et al. (1979) reported the amino terminal sequence (20 amino acids) of the α-subunit of Torpedo marmorata. More recently the identification of the messenger RNA for the AChR has allowed the cloning of complementary DNA(cDNA) and subsequent nucleotide sequencing of the α-chain in Torpedo marmorata (Sumikawa et al., 1982; Devillers-Thiery et al., 1983) and of all four chains in Torpedo californica (Ballivet et al., 1982; Noda et al., 1982, 1983a,b). In addition Noda et al. (1983c) have cloned cDNA for the

α-subunit precursor of calf skeletal muscle AChR. Nucleotide sequencing of the mammalian DNA has revealed marked sequence homology with their Torpedo counterparts.

The receptor contains carbohydrate as shown by the interaction with plant lectins (Meunier et al., 1974) and this property has been utilised in the purification of mammalian muscle AChR (Shorr et al., 1978; Brockes and Hall, 1975). Polyacrylamide electrophoresis gels of purified AChR stain with reagents specific for carbohydrate (Shorr et al., 1978; Lindstrom et al., 1979) and the carbohydrate composition of Torpedo californica AChR has been analysed by gas-liquid chromatography (Lindstrom,1979a; Mattson and Heilbronn, 1975; Raftery et al., 1976). The carbohydrate content of Torpedo receptor represents 4-7% of the total receptor weight, with all of the individual subunits possessing glyco-moieties (Vandlen et al., 1979; Turnbull et al., 1984). There is general agreement on the presence of mannose, galactose and N-acetylgalactosamine (Heilbronn et al., 1974; Lindstrom et al., 1979a; Vandlen et al., 1979). Neuraminidase treatment of the receptor, affecting exclusively the γ and δ subunits, has suggested the presence of sialic acid residues in these subunits (Criado and Barrantes, 1982) and a recent direct determination has confirmed the presence of about one mole of sialic acid per mole of receptor (Bersinger et al., 1983).

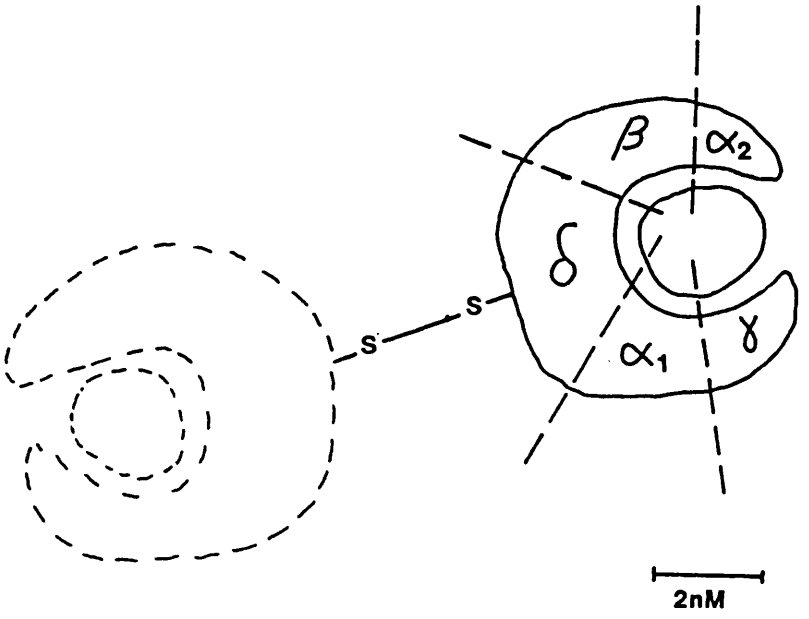
The possible functional involvement of a phospholipid component is suggested by the effect of phospholipase A treatment on the binding of acetylcholine and carbamylcholine by the receptor (Eldefrawi et al., 1972; Hanley, 1978; Andreason et al., 1979). The lipid environment of the receptor has been reported to modulate its functional properties (Chang and Bock, 1979; Andreason et al., 1979) and a

fraction of immobilised lipid has been reported to occur in the vicinity of the AChR (Marsh et al., 1981; Ellena et al., 1983). More detailed studies of the influence of the lipid environment on the AChR have been allowed by reconstitution of the receptor into artificial lipid membranes (Epstein and Racker, 1978; Anholt et al., 1982). Ion flux in such systems depends on the presence of neutral lipids (Dalziel et al., 1980) and charged natural phospholipids (Ochoa et al., 1983). The transition of the receptor between low and high affinity states is also dependent on the lipid constitution of the reconstituted membrane (Heidmann et al., 1980; Criado et al., 1984).

The structural characteristics of membrane-bound AChR

The AChR is an integral membrane protein, spanning the post-synaptic membrane, and extending out approximately 1.5nm from the intracellular surface and almost 5.5nm from the extracellular surface (Ross et al., 1977; Huang, 1979; Tarrab-Hazdai et al., 1978). The use of electron microscopy, combined with single-particle image averaging, indicates that the two α-subunits are diametrically opposed with about 5nm separating them (Zingsheim et al., 1982). Cross-linking studies demonstrate that predominantly the α and δ chains are cross-linked to α-toxin (Karlin, 1980; Hucho, 1981). Scanning electron microscopy of diamide-induced trimers of AChR in receptor-rich membranes has given an indication of the spatial arrangement of the β and δ subunits (Wise et al., 1981). From such data a schematic representation of the subunit arrangement and topography of the membrane-bound AChR can be made (Figure 5). The orientation of specific subunits with respect to the membrane has been investigated by a variety of techniques, indicating that parts of all four

FIGURE 5. A schematic representation of the subunit topography of the membrane-bound AChR protein.



subunits are exposed on both the extracellular and cytoplasmic surfaces. The 43000 dalton protein is, however, exposed on only the cytoplasmic face (Cohen et al., 1980; Karlin, 1980; Wennogle et al., 1981; Sargent et al., 1984).

Pharmacology of AChR in it's purified and membrane-bound forms

Although AChR's are classified into muscarinic and nicotinic types, the fact that acetylcholine binds to both types of receptor, as well as to acetylcholinesterase, choline acetyltransferase and certain storage proteins, indicates that there are similarities in the active sites of these proteins. Because the action of many neuroactive drugs is attributable to their structural similarities to the neurotransmitter it is not unusual to find a cholinergic drug with more than one site of action; the primary target usually being the receptor for which it has the highest affinity. Thus, classification of drugs into muscarinic and nicotinic groups is dependent on their affinities for the two types of AChR, and hence a "drug profile" for the nicotinic AChR can be built up. From this it follows that in order to determine if the AChR protein has been altered by solubilisation and purification it is necessary to measure its affinity for cholinergic agonists and antagonists before and after such treatments. The binding characteristics of cholinergic ligands can be measured in a number of ways (Colquhoun, 1979; Conti-Tronconi and Raftery, 1982). On the basis of all of the available methods for determination of the detailed binding characteristics of both electric fish and skeletal muscle receptors, there is generally good agreement between the characteristics of soluble and membrane-bound receptors (for reviews see Dolly, 1979; Colquhoun, 1979), although the interconversion of agonist affinity states induced by desensitization is lost on

dissolution of the membrane by neutral detergents or in sodium cholate (for review see Conti-Tronconi and Raftery, 1982).

The action of antagonists is generally considered to result from simple competitive binding to the acetylcholine binding site. Physiologically, therefore, the α-neurotoxins act as antagonists, although the half-time of the blockade reversal is up to several days (Lee, 1972). Radiolabelled α-BGT binds to the AChR with regular second-order kinetic behaviour, and has a similar K_D for both partially purified and membrane-bound rat muscle receptor (Dolly, 1979). A competitive relationship is observed between the binding of cholinergic effectors and the α-toxins to the AChR, with each AChR macromolecule possessing two ligand/toxin binding sites. (for reviews see Fulpius, 1976; Heidmann and Changeux, 1978).

A number of substances are known which block the physiological response to acetylcholine. Local anaesthetics are thought to work by binding to the ion-channels. Histrionicotoxin, an alkaloid isolated from the Colombian arrow-poison frog, Dendrobates histrionicus (Daly et al., 1971) can also reversibly block the acetylcholine-mediated ion conductance at the neuromuscular junction, possibly via a direct block on the AChR ion channel (for review see Conti-Tronconi and Raftery, 1982).

Junctional and extrajunctional AChR

The distribution of AChR in the muscle cell membrane has been investigated by both the iontophoretic mapping of acetylcholine, and by autoradiography of bound α-BGT (for review see Fambrough, 1979).

In the embryonic myotube, prior to the onset of innervation, the receptor protein is widely distributed over the cell surface

(extrajunctional receptor). As development of the neuromuscular junction progresses the receptor becomes progressively restricted to the junctional region (junctional receptor).

In adult innervated muscle the receptor is present only in the end-plate region (Dolly, 1979). If a mature, innervated muscle is denervated, however, the extrajunctional regions acquire receptors, producing a density which is less than the junctional area, but greater than embryonic muscle receptors (for review see Fambrough, 1981).

Extrajunctional and junctional receptors in situ differ in a number of ways.

1. Embryonic extrajunctional receptor on cultured cells has been shown to exhibit significant lateral mobility, with a diffusion coefficient of 1 - 3 x 10^{-9} cm^2/s at 37°C (for review see Fambrough, 1979), whereas the junctional receptor is immobile.
2. The rate of turnover of extrajunctional receptor from both embryonic and denervated adult muscle is considerably higher than that of junctional receptor in adult muscle (average receptor lifetimes of 6 - 35h and >120h respectively; for review see Fambrough, 1979).
3. The ion channel of the receptor from adult rat and chick muscle possesses a mean channel open time 3 - 5 fold longer than that of the extrajunctional receptor from denervated adult muscle (Katz and Miledi, 1972; Neher and Sakmann, 1976).

Purification of AChR from innervated and denervated adult rat muscle has demonstrated that junctional and extrajunctional receptors are remarkably similar in subunit composition, and are indistinguishable by gel filtration and sucrose density centrifugation (Brockes and Hall, 1975; Froehner et al., 1977b; Nathanson and Hall, 1979). Nevertheless purified junctional and extrajunctional receptors from rat muscle have been reported to display three significant differences.

1. They have slightly different isoelectric pHs (Brockes and Hall, 1975).
2. They exhibit different cholinergic drug sensitivities (Beranek and Vyskocil, 1967; Brockes and Hall, 1975).
3. Extrajunctional receptor may display more antigenic determinants than junctional receptor (see below).

The available evidence to date suggests that the biochemical and physiological differences between junctional and extrajunctional receptors result from post-translational modifications, such as phosphorylation and glycosylation (for review see Barnstaple et al., 1983). It is possible that the receptor undergoes such modification before being assembled in the membrane (Changeux and Danchin, 1976). The receptor will also be affected by its local environment, and in particular the lipid composition of this environment (Hall, 1981).

The AChR of muscle cells in culture

Tissue culture has been used to study smooth, striated and cardiac muscle, but has been most fruitful in the case of striated, skeletal muscle.

Multinucleated myotubes can be formed in culture from myoblasts obtained from embryonic muscle, or from satellite cells from adult

muscle biopsies, or from established myoblast cell lines. Associated with the appearance of fused fibres in the culture are increases in the specific activity of a number of enzymes, and the accumulation of contractile proteins (for review see Schubert et al., 1973). At the time of myoblast fusion, AChR, detected either by electrophysiological response to acetylcholine or by ^{125}I -α-BGT binding, begins to appear in the culture (Fambrough and Rash, 1971; Patrick et al., 1972; Vogel et al., 1972). The AChR may be elaborated from a pool pre-existing in the myoblast (Teng and Fiszman, 1976), or by de novo synthesis after fusion has occurred (Merlie et al., 1975). Once the fusion process is complete the number of receptors on the surface of a muscle fibre represents a steady state determined by the relative rates of synthesis and degradation (Fambrough, 1979).

In cultured chick myotubes synthesis of AChR occurs in less than 30 minutes, but the receptors undergo a 3 hour transit time before being inserted into the membrane (Devreotes and Fambrough, 1976). A substantial fraction of newly synthesised receptor is located in the Golgi apparatus, where it resides for about 2 hours (Fambrough and Devreotes, 1978; Merlie and Sebbane, 1981; Bursztajn and Fischbach, 1984). Each subunit is translated from a separate mRNA (Anderson and Blobel, 1981) with co-translational insertion of each subunit into the rough endoplasmic reticulum (RER) membrane. Concomitant with this the subunits are also glycosylated. The subunits are also translated with signal peptides that govern their subsequent orientation in the membrane (for review see Anderson and Blobel, 1983). The AChR subunits are not assembled with one another into a functional complex immediately upon incorporation into the RER membrane. Assembly rather appears to be a fairly lengthy post-translational process. Post-translational modification of the α-subunit to form the high-

affinity α-BGT binding site occurs over a period of about 30 minutes after transport of the subunit out of the RER into the Golgi apparatus (Merlie and Sebbane, 1981). Quaternary assembly of the subunits may take at least as long as acquisition of the toxin-binding site (Merlie et al., 1983). The incorporation of new receptors into the plasma membrane is blocked by inhibitors such as 2,4 - dinitrophenol which interfere with ATP synthesis. Incorporation is also dependent on temperature in homeotherms, and does not occur at a significant rate below 25°C in cultured chick skeletal muscle (Fambrough, 1979). Treatment of cultured chick muscle with a specific inhibitor of N-glycosylation, tunicamycin, has been reported to result in a ten-fold reduction in the number of surface ^{125}I -α-BGT binding sites. Bound toxin was also released at a more rapid rate than in control cultures (Prives and Olden, 1980). Tunicamycin also blocks maturation of the α-BGT binding site on the α-subunit (Merlie et al., 1981).

The degradation rate of AChR can be approximated by measuring the rate of degradation of ^{125}I -α-BGT after it has bound to the receptor. From these data the average receptor lifetimes for extra-junctional AChR on cultured muscle has been calculated at about 17 - 19 hours (for review see Pumplin and Fambrough, 1982). The degradation process in chick muscle cultures is sensitive to protein content and pH of the culture medium, and is exquisitely sensitive to temperature (Devreotes and Fambrough, 1975). Metabolic inhibitors which interfere with ATP synthesis rapidly block or slow the degradation process, with a slower retardation being given by inhibitors of protein synthesis (Fambrough, 1979). Degradation of the AChR-toxin complex occurs via a process of internalisation followed by proteolytic degradation in secondary lysosomes (Devreotes and Fambrough, 1975; Axelrod et al., 1976). Many different types of experiments have shown that the

processes of AChR synthesis and degradation are not directly coupled (for review see Pumplin and Fambrough, 1982).

Newly synthesised and incorporated AChR in cultured muscle membranes may remain in diffuse distribution, or partition into discrete clusters (Vogel et al., 1972; Sytkowski et al., 1973; Fischbach and Cohen, 1973; Prives et al., 1976; Bloch, 1979). AChR clusters are typically round or ovoid patches 5 - 10μm across, consisting of laterally immobile AChR with a density 2 - 3 fold lower than those at mature synapses (Sytkowski, 1974), and 10 fold higher than diffusely distributed receptors (Axelrod et al., 1976). Clusters may be found either juxtaposed to the substrate or on the free surface of the cell. In rat myotubes the localisation is primarily on the ventral surface, and is within broad regions of close cell to substrate contact (Bloch and Geiger, 1980). It is associated with a number of other cell membrane associated components (for review see Fambrough, 1984). Clusters of receptors have a half-time in the membrane similar to that of non-clustered receptors (Schuetze et al., 1978). Diffuse and clustered AChRs do not readily interconvert in short times, but can be induced to do so by treating cultures with sodium azide, or with medium depleted of calcium (Bloch, 1979). Autoradiographic studies of receptor distribution in embryonic rat and chick skeletal muscle in vivo suggest that AChR clusters do not occur on myotubes prior to innervation and occur only at neuromuscular connections thereafter (Burden, 1977; Bevan and Steinbach, 1977). The formation of clusters in vitro may, therefore, be a manifestation of synaptogenic mechanisms operating in the prolonged absence of innervation (Fambrough, 1979).

The AChR and Myasthenia Gravis

Myasthenia gravis is a neuromuscular disorder which pursues a remitting and relapsing chronic course and is principally characterised by muscle weakness (Simpson, 1978a,b). The disease has a prevalence which is within the range of 1:10000 to 1:50000 of the population. The age of presentation ranges from early childhood to extreme old age. The incidence of myasthenia is higher in females than males, with a ratio of 6:4. In those presenting under 40 years, females predominate, while in those presenting above this age, males predominate, with modal values for the two sexes being in the third and seventh decades respectively (Osserman and Genkins, 1971). The initial symptoms may present insidiously or may arise suddenly if precipitated by such factors as emotional disturbance, infection of the upper respiratory tract or anaesthesia. At first the weakness may affect any muscle, but certain ones are likely to be more affected than others. For example, weakness of the extraocular muscles is common, causing diplopia. Involvement of the elevators of the upper eyelids gives rise to ptosis. Other facial muscles may also be affected, and there is often weakness of bulbar muscles causing difficulty in swallowing and speaking.

The disease may undergo spontaneous remission although this is uncommon, and it is more usual for the weakness to spread to muscles other than those originally affected. A characteristic feature of myasthenia is the fact that the weakness is aggravated by exertion and improved by rest.

Other clinical disorders may be associated with myasthenia. Of these, by far the most important is a thymoma which is present in about 10% of cases. A third of these tumours are malignant, but

metastases are uncommon. Disorders of the thyroid gland are also associated with myasthenia, being found in about 9% of males and 18% of females (Simpson, 1958; Downes et al., 1966). The most common of these disorders is thyrotoxicosis, but myxoedema, non-toxic goitre and Hashimoto's thyroiditis may also occur. A variety of other diseases have been reported in conjunction with myasthenia, and include rheumatoid arthritis, Sjogren's disease, pernicious anaemia, epilepsy and psychotic illness (Simpson, 1974).

The first clear description of myasthenia gravis was given by Dr Thomas Willis in 1672 in his book "De Anima Brutorum". In the English version (published in 1683) he describes persons who:

'are distempered with Members very much loosened from their due vigour and strength, and with a languishing of their Limbs; so that though they are well in their stomach, and have a good and laudable pulse and wine, yet they are as if they were enervated, and cannot stand upright, and dare scarce enter upon local motions, or if they do, cannot perform them long: yea some without any notable sickness, are for a long time fixed in their Bed, as if they were every day about to dye; whilst they lie undisturbed, talk with their Friends, and are chearful, but they will not, nor dare not move or walk; yea they shun all motion as a most horrid thing ..... wherefore the sick are scarce brought by any perswasion to try whether they can go or not. Nevertheless, those labouring with a want of spirits, who will exercise local motions as well as they can, in the morning are able to walk firmly, to fling about their Arms hither and thither, or to take up any heavy thing, before noon the stock of spirits being spent, which had flowed into the Muscles, they are scarce able to move Hand or Foot'.

A further two centuries passed before one finds another description suggestive of myasthenia. This was in a paper published in 1877 by Sir Samuel Wilks. Two years later a fuller description was given by a German physician, Erb, and over the next 15 years more isolated reports followed. The understanding of the disease was significantly advanced by Jolly (1895) who was able to show that muscle strength was normal initially, but then declined rapidly in patients (other workers subsequently demonstrated that electrical stimulation of the muscle fibres directly was effective in restoring contractions), and was the first to use the name myasthenia gravis (in fact myasthenia gravis pseudoparalytica) to describe the disease. Jolly also noted its similarity to curare poisoning.

The observations of Jolly, together with the findings much later of the beneficial effects of anticholinesterase drugs in the disease (Walker, 1934), and the demonstration of normal nerve action potentials (Alajouanine et al., 1959) all suggested that the neuromuscular junction was the site of the abnormality in myasthenia gravis. A number of defects in neuromuscular transmission are theoretically possible in myasthenia, which may be classified as follows:

1. Presynaptic

   a. Failure of impulse propagation in axon terminal.

   b. Impaired synthesis of acetylcholine.

   c. Faulty packaging mechanism in vesicles.

   d. Impaired release of acetylcholine.

   e. Release of a "false" transmitter.

2. Intrasynaptic

   a. Excess diffusion of acetylcholine away from the primary

synaptic cleft.

b. Diffusion barrier.

3. Postsynaptic

a. Combination of a circulating blocking agent with AChR.

b. Depletion of AChR.

c. Desensitisation of AChR.

d. Increased acetylcholinesterase activity.

Many different, and widely differing, techniques have been used to determine the nature of the specific lesion in myasthenia gravis. The evidence of morphological, pharmacological and neurophysiological studies is summarised below.

Morphological studies

Using light microscopy, in combination with methylene blue "vital" staining, or cholinesterase staining, Coërs and Woolf (1959) found two types of abnormality of the motor nerve endings which they termed "dystrophic" and "dysplastic".

Dystrophic junctions were characterised by branching of a single motor axon so as to form multiple end-plates on the same muscle fibre. Dysplastic junctions were those in which the end-plates were abnormally lengthened on the surface of the fibre. Similar findings to those of Coërs and Woolf were obtained by MacDermott (1960) and Reske-Nielsen et al. (1965). A further important observation was that both types of neuromuscular abnormality could be found on muscle fibres without any microscopic evidence of degeneration, suggesting that the neuromuscular lesion was a primary event.

A more detailed examination of the myasthenic neuromuscular junction by electron microscopy has shown a generalised simplification of structure. A widening of both primary and secondary clefts was observed, with a reduction in the number of synaptic clefts also being noted (Zacks et al., 1961; Santa et al., 1972). In contrast to these findings, the fine structure of the axon terminal appeared well preserved in myasthenia, with normal numbers and sizes of synaptic vesicles being noted by Johnson and Woolf (1965) and Santa et al. (1972). One pre-synaptic change which was clearly evident in some junctions studied by both Zacks et al., and Johnson and Woolf was a reduction in the size of the terminal axonal expansion. As the axon retracted, its place was taken by an extension of Schwann cell cytoplasm. A pictorial summary of the morphological features of the myasthenic neuromuscular junction is shown in Figure 6.

Early investigations performed at post mortem on the muscle fibres of myasthenic patients revealed three categories of changes (Russell, 1953).

Type I: An acute change in which the fibres underwent coagulative necrosis and became swollen, subsequently losing their nuclei and myofibrils. There was a pronounced infiltration of inflammatory cells in and around the fibres, in which polymorphonuclear leukocytes and macrophages predominated.

Type II: This change was concerned with the formation of lymphorrhages (collections of small lymphocytes around single muscle fibres undergoing atrophy).

Type III: A simple atrophy affecting single muscle fibres or groups of fibres. The characteristic eosinophilia of the sarcoplasm was

FIGURE 6. (After Lindstrom, 1979)

A Diagrammatic representation of a normal neuromuscular junction.

(1) Acetylcholine containing vesicles in the nerve ending.

(2) The presynaptic membrane of the nerve.

(3) The intersynaptic space across which acetylcholine must diffuse after release through specific regions in the presynaptic membrane.

(4) Folds in the postsynaptic membrane of the muscle;the acetylcholine receptors are concentrated at the tips and are close to the sites of acetylcholine release.

B Diagrammatic representation of a neuromuscular junction in chronic EAMG and human myasthenia gravis.

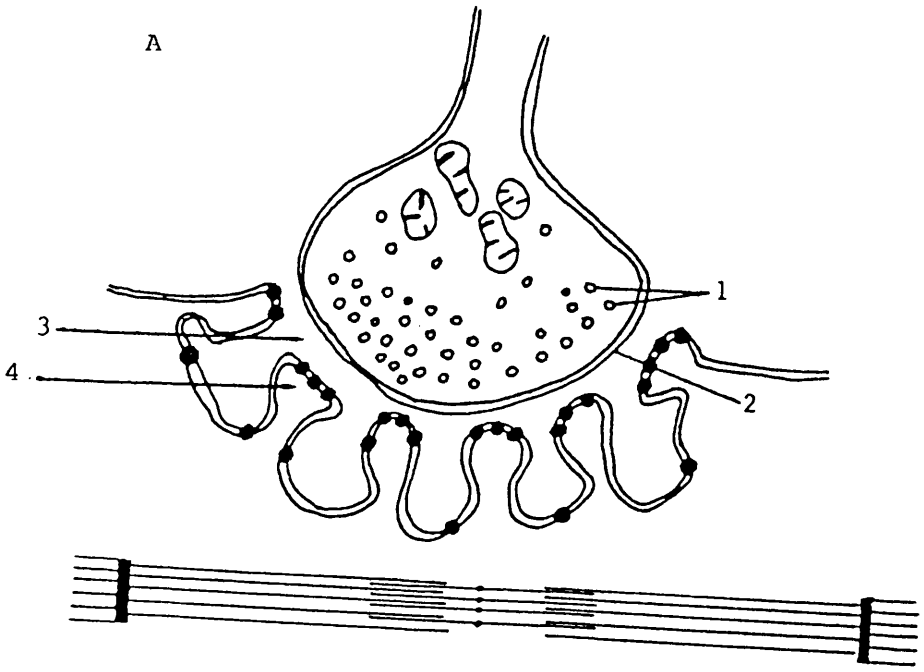
(1) The folded structure of the postsynaptic membrane is highly simplified and contains a greatly reduced number of acetylcholine receptors.

(2) Many of the acetylcholine receptors that remain have antibody bound to them.

(3) Complement-mediated focal destruction of the postsynaptic membrane causes loss of acetylcholine receptors through shedding of complexes of antibody,acetycholine receptors and complement into the intersynaptic space.

(4) Antigenic modulation of the acetylcholine receptor causes loss of receptors independent of complement.This may proceed through endocytosis of the receptors aggregated by antibodies.

(5) Enhanced synthesis of the acetylcholine receptors may partially compensate for the loss of receptor from the membrane.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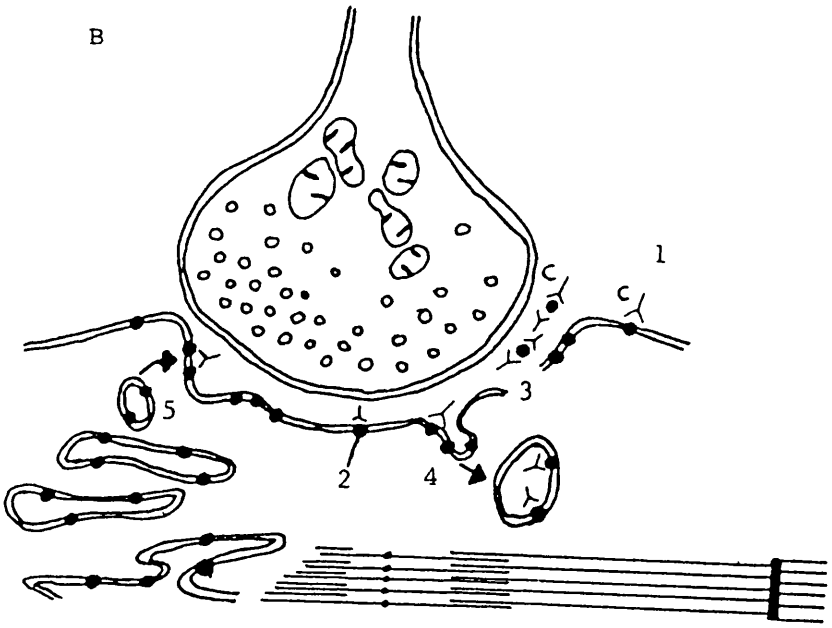
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retained, and the nuclei remained at the periphery of the fibres. No loss of cross-striations was noted and there was no infiltration by inflammatory cells.

More recently Werneck (1982) found that fresh-frozen sections from 15 of 17 muscle biopsies of myasthenic patients were abnormal. Of these, 7 had type III atrophy, 4 showed lymphocyte infiltration and one had fibre necrosis with phagocytosis. Eleven of the biopsies showed evidence of denervation. There was a direct correlation between disease duration and the severity of histological abnormality.

Pharmacological studies

Pharmacological tests of myasthenic patients have shown that they are unusually susceptible to the effects of curare and d-tubocurarine. They are resistant to the prompt depressant action of acetylcholine when injected intra-arterially, and are also resistant to the administration of decamethonium. Finally their symptoms are alleviated by anti-acetylcholinesterases (for review see Simpson, 1974).

Neurophysiological studies

Neurophysiological studies of myasthenic patients have shown that the action potential generated by the nerve is normal. Intracellular recordings of the electrical activity of the muscle fibre, however, have shown that the miniature end-plate potentials (MEPPs) are reduced in size (Elmquist et al., 1964) and that myasthenic motor end-plates in vitro are much less sensitive to iontophoretically applied acetylcholine than normal end-plates (Rash et al., 1976). Multi-electrode recordings from two or more muscle fibres from the same motor unit during a voluntary contraction showed a greater time

delay between the two impulses in myasthenic muscle fibres than in normal fibres (Ekstedt and Stalberg, 1967). This is attributed to the delay in onset of end-plate potentials resulting from the diminished size of the MEPPs. Measurements of whole muscle subjected to repetitive supramaximal stimuli of the nerve show that a significant decrement is observed in myasthenia (Desmedt, 1966). The decremental response is consistent with the observed physical fatiguability of myasthenic patients, and the experimental finding of reduced MEPPs. A pre-synaptic origin for the physiological abnormalities is discounted by the finding that the acetylcholine content of myasthenic muscle is actually higher than in control muscle (Ito et al., 1976) and that 2 - 5 times as many quanta of acetylcholine are released at myasthenic motor nerve terminals than at normal terminals (Cull-Candy et al., 1980). The abnormalities may, however, be adequately accounted for by the reduction in the number of AChRs in the post-synaptic membrane of the myasthenic junction which has been reported (Ito et al.,1978).

The immunology of myasthenia gravis

It has been found that one in seven children born to myasthenic mothers shows transient symptoms of myasthenia which disappear after a few weeks of life. This observation suggests that a blocking agent crosses the placenta from the mother to the child in these cases, and is effective in blocking neurotransmission. The observations on neonatal myasthenia, and the superficial resemblance of myasthenic weakness to that of curare intoxication prompted numerous attempts to demonstrate the existence of a humoral blocking agent in myasthenia. In 1934 Mary Walker, best known for her introduction of anticholinesterase therapy in myasthenia, reported that ptosis could occur

following release of a tourniquet that had prevented return of venous blood from the exercised limb of a myasthenic patient. In 1944 Wilson and Stoner applied serum from a myasthenic patient to a frog nerve-muscle preparation, and demonstrated that it had a blocking effect following stimulation of the nerve. The blocking effect has, however, proved to be extremely variable. Nastuk et al. (1959) only occasionally found a blocking effect, while Parkes and McKinna (1966) observed significant reductions in the contractile responses of indirectly stimulated rat diaphragm muscles, and were able to reverse the decrement with anticholinesterase drugs.

In 1960 Simpson proposed that myasthenia gravis is an autoimmune disorder. The subsequently accumulated evidence supporting an autoimmune basis for the disease can be summarised as follows:

1. Myasthenia resembles systemic lupus erythamatosis (SLE), a known autoimmune disorder, in being more common in females and in having the highest incidence of onset in the third decade.
2. Myasthenia is often associated with other autoimmune disorders such as rheumatoid arthritis, SLE, Sjogren's syndrome and Raynaud's syndrome.
3. The infiltration of lymphocytes within some of the myasthenic muscles is suggestive of an autoimmune disorder.
4. There is a high incidence of thymic abnormalities (thymic hyperplasia and thymoma) in myasthenia, and it is known that the thymus gland has an important role in immune mechanisms (for review see Yunis et al.,1971).
5. At about the same time that Simpson put forward his hypothesis, Strauss et al. (1960) reported the presence in a

significant proportion of myasthenic patients of circulating antibody to the A - band of striated muscle fibres. This finding has since been confirmed in many laboratories, and also extended by the demonstration that nearly every myasthenic patient with a thymoma carries these antibodies.

6. Daily injections of immunoglobulin from myasthenia patients intraperitoneally into mice for periods of 10 - 14 days resulted in the animals acquiring a number of myasthenic features (Toyka et al., 1975).
7. The development of transient myasthenia in the infants of affected mothers is indicative of a neurohumoral agent traversing the placenta. It is known that IgG can be transported from mother to foetus via the placenta.
8. Nearly all patients with myasthenia gravis carry serum antibodies to AChR (Lindstrom et al., 1976d).
9. Immunosuppressant drugs are found to be beneficial in the treatment of myasthenia.
10. Plasma exchange can induce a short-term remission in acquired myasthenia.
11. Experimental models can be produced by immunising animals with preparations of muscle, thymus or AChR.

The evidence for myasthenia's being an immune disorder principally affecting the AChR is now overwhelming, and is detailed more fully below.

Animal models of myasthenia gravis

1. The Goldstein model: In 1966 Goldstein and Whittingham showed that it was possible to induce an autoimmune inflammatory response

when animals were injected with a saline extract of thymus or striated muscle. These animals also exhibited a transient block of neuromuscular conduction which had several features in common with myasthenia gravis. Goldstein and Manganaro (1971) identified two polypeptides from the normal thymus, each having a molecular weight of 7000 daltons. One of these, thymin, was considered responsible for inducing the neuromuscular block, while the other, thymotoxin, was thought to cause the inflammatory responses in the muscles which the experimentally-treated animals commonly showed. This led Goldstein et al. (1976) to present the hypothesis that thymin imbalances are involved in the aetiology of autoimmune diseases.

2. Experimental autoimmune myasthenia gravis (EAMG): An early observation in the course of raising antibodies for the immunochemical characterisation of purified AChR was that rabbits immunised repeatedly with receptor in adjuvant developed profound muscle weakness (Patrick and Lindstrom, 1973; Sugiyama et al., 1973). This observation led to the EAMG animal model of myasthenia gravis (Lennon et al., 1975). EAMG is readily induced in some mammals by a single intradermal inoculation of microgram quantities of AChR purified from either electric fish or skeletal muscle, and resembles spontaneously occurring myasthenia in almost every respect (for review see Lennon, 1976). Since the original report EAMG has been induced in many other animals including rats, mice, guinea pigs, goats, monkeys, frogs (for review see Lindstrom, 1979), chicken, sheep (Barkas et al., 1978) and dogs. EAMG is caused only by AChR, rather than any minor contaminant of the receptor preparation (Claudio and Raftery, 1980).

Immunisation of Lewis rats with purified AChR plus adjuvant or pertussis results in a biphasic form of EAMG, consisting of an acute

followed by a chronic phase. Figure 6 shows the characteristics of the chronic phase. The acute phase occurs about 8 - 11 days post inoculation, and is associated with a massive invasion of the end-plate region by mononuclear inflammatory cells which destroy the post-synaptic membrane (Engel et al., 1976a,b). A human counterpart of this acute phase has not been recognised, but the occurrence of lymphorrhages and the occasional association of polymyositis with myasthenia gravis (Johns et al., 1971) may be evidence of a similar cellular contribution to the pathogenesis of myasthenia in man. After day 12 the rats recover and inflammatory cells disappear. Around day 30 a chronic phase of weakness ensues which usually progresses to death over several weeks. In the chronic phase, the post-synaptic membrane is highly simplified, with loss of its intricate folding and a widening of the synaptic gap (Engel et al., 1976a,b). This picture is identical with the appearance of human neuromuscular junctions in established myasthenia gravis (see Figure 6). A further similarity is that EAMG can be induced by passive transfer by injection of serum from rats with chronic EAMG (Lindstrom et al., 1976b; Lennon et al., 1978; Engel et al., 1979). Passively transferred EAMG is similar to the acute phase of the experimental disease (Engel et al., 1979). The major points of similarity between myasthenia gravis and chronic EAMG are summarised in Table 2.

Analysis of the serum of animals with EAMG has demonstrated the presence of circulating AChR antibodies to both the foreign immunogen (Patrick and Lindstrom, 1973) and to the host AChR (Aharonov et al., 1977). Additionally the presence of antibodies bound to AChR in the muscle membrane has been demonstrated (for review see Lindstrom, 1979). The serum concentration of anti-AChR antibodies is lower during acute EAMG, and the degree of cross-reaction with muscle AChR

TABLE 2. Similarities between myasthenia gravis and it's animal model,experimental autoimmune myasthenia gravis (EAMG).

(Adapted from Lennon, 1976).

Clinical	Muscle weakness with easy fatigability. Temporary improvement by rest and by anti-cholinesterase drugs. Muscles of the head and neck,upper limbs and respiration primarily affected.
Electrophysiological and Pharmacological	Decrementing response of muscle during repetitive motor nerve stimulation at low rates. Post-activation facilitation followed by exhaustion induced by rapid repetitive stimulation. Electrophysiological defect repaired by anticholinesterases. Amplitude of miniature end-plate potentials reduced,but with normal numbers of transmitter quanta released by a nerve impulse. Curare sensitivity increased. Number of ^{125}I-α-BGT binding sites at the neuromuscular junction greatly reduced.
Ultrastructural	Appearance of motor endplates in EAMG indistinguishable from those of myasthenic patients (Figure 6.).
Immunological	Antibodies and cellular immunity to AChR demonstrable.

is lower than that at later stages (Lindstrom et al., 1976a,b).

The autoimmune response in myasthenia gravis

Following the demonstration of circulating anti-receptor antibodies in EAMG similar antibodies were sought in human myasthenic sera using a variety of techniques (Table 3).

Mittag et al. (1976) showed that the highest percentage of positive results was found when immunoprecipitation of IgG -α-BGT - labelled AChR was used, and this assay has now become standard practice (for review see Newsom-Davis and Vincent, 1982) with the parameters of the assay having been optimised (Carter et al., 1981).

Circulating anti-AChR antibodies are present in at least 90% of clinically diagnosed myasthenics (Lindstrom et al., 1976a; Monnier and Fulpius, 1977). The level of antibody is, however, generally found not to correlate with disease severity (Vincent and Newsom-Davis, 1979; Barkas et al., 1979). Nevertheless, Lefvert et al. (1978) and Carter et al. (1980) found good correlation when the antibody titre was compared with an individual's clinical progress. Patients with ocular myasthenia only have generally lower titres than more severe cases (Lindstrom et al., 1976a; Lefvert et al., 1978). Patients with thymoma tend to have higher titres, reflecting the fact that the thymus is a site of anti-AChR antibody production in some myasthenic patients (for review see Newsom-Davis and Vincent, 1982). Among those with generalised disease, titres can range over 2 or 3 orders of magnitude for patients with similar disease severity. Factors such as age, sex, duration of disease, or treatment with steroids or thymectomy, do not appear to influence anti-receptor antibody titres in myasthenic patients compared as groups. Antibodies

TABLE 3. Methods for the detection of anti-AChR antibodies in myasthenia gravis.

SOURCE OF AChR	METHOD	% POSITIVE	REFERENCE
Denervated rat muscle extract	Inhibition of α- BGT binding	33	Almon et al., 1974.
Normal rat muscle extract	" " "	0	Almon and Appel, 1975.
Torpedo marmorata membranes	Inhibition of cobrotoxin binding	62	Lefvert and Bergstrom, 1977.
Denervated rat muscle membranes	Inhibition of α- BGT binding	0 - 8	Mittag et al., 1976.
Denervated rat muscle extract	Inhibition of AChR binding to Con-A Sepharose	66	" " "
Normal human muscle end-plates in cross-section	Inhibition of indirect immunoperoxidase labelling of AChR	44	Bender et al., 1975.
Denervated human muscle fibres in cross-section	" " "	75	Ringel et al., 1975.
Torpedo californica AChR	Complement fixation	80	Aharonov et al., 1975.

TABLE 3 cont.

SOURCE OF AChR	METHOD	% POSITIVE	REFERENCE
Denervated rat muscle extract	Immunoprecipitation of α- BGT-labelled AChR from crude muscle extracts with anti-IgG	70 - 85	Appel et al., 1975. Mittag et al., 1976.
Normal rat muscle extract	" " "	0	Appel et al., 1975.
Human limb muscle extract	" " "	91	Lindstrom, 1977.
Human muscle extract	" " "	75 - 93	Monnier and Fulpius, 1977. Ito et al., 1978. Lefvert et al., 1978.

have been detected in neonatal myasthenics (Lefvert et al., 1978) and also in the cerebrospinal fluid of some patients (Bergstrom et al., 1978; Keesey et al., 1978). They have also been detected in the serum of rheumatoid arthritis patients treated with D-penicillamine who developed symptoms of myasthenia (Vincent et al., 1978; Argov et al., 1980). These symptoms were responsive to anticholinesterase drugs, and disappeared on withdrawal of D-penicillamine.

Other autoantibodies in myasthenia gravis

Various autoantibodies other than anti-AChR antibodies are found with increased frequency in myasthenic patients. Some of these antibodies are associated with immune diseases, but their incidence is much higher than clinical symptoms of other disorders. The incidence of autoantibodies other than anti-AChR antibodies in myasthenia is summarised in Table 4.

The overall incidence of anti-striated muscle antibody is about 40% of myasthenic patients. Investigations by Beutner et al. (1962) showed that antibodies could be classified as either anti-striated muscle only (anti-SM) or anti-striated and cardiac muscle (anti-SH). It was soon realised that almost all patients with thymoma have anti-SM antibodies (Van der Geld et al., 1963; Oosterhuis et al., 1968). Anti-SM antibodies are also associated with the HLA - A antigen (Feltkamp et al., 1974) and the presence of DR2 (Compston et al., 1980).

Characterisation of the immune response in myasthenia gravis

The lack of correlation between anti-AChR antibody titre and disease severity implies that myasthenic sera contain a heterologous population of anti-receptor antibodies, not all of which are pathogenic. This is also true of sera from animals with EAMG. Such a

TABLE 4. Incidence of autoantibodies other than anti-AChR antibody in myasthenia gravis compared to the normal population.

(Adapted from Vincent and Newsom-Davis, 1982)

Autoantibody	Percentage of patients possessing the autoantibody.	
	Myasthenia gravis	Normal controls
Thyroid	12-43	5-22
Antinuclear factor	9-75	4-9
Gastric parietal cell	0-23	3-13
Striated muscle	20-51	0-7

heterogeneous antibody population would be expected to differ not only in their antigenic specificity, but also in their binding affinity, and ability to interact with other immune effectors such as complement and phagocytes. Thus disease state may be better related to specific subpopulations of anti-AChR antibodies.

Three basic types of technique have been used in elucidating the characteristics of anti-AChR antibodies, and the nature of AChR antigenicity and immunogenicity.

Firstly the effects of myasthenic sera, or sera from animals immunised with whole native AChR, on a range of biochemical reactions of the receptor, such as ligand binding, ion-channel function, and turnover of the receptor in situ, have been studied. The physical, immunological and kinetic properties of the antibody population itself have also been examined.

Secondly, molecular modifications of the purified AChR have been made, and used both for immunisation of experimental animals, and for cross-reaction studies with antisera raised against native receptor. The rationale behind such an approach is that modification of the AChR, followed by pharmacological and immunological analysis may lead to the isolation and characterisation of small fragments of the molecule which are responsible for the specific pathological myasthenic activity of the receptor (Fuchs et al., 1981).

Thirdly, monoclonal antibodies (mAbs) to AChR have been prepared. Such antibodies have the potential to be excellent probes for the AChR, as they have specificities that can be strictly defined. A number of workers have begun to map the binding characteristics of panels of anti-AChR mAbs. These mAbs have also been used as

competitive ligands to map the antibody specificities present in heterogeneous antisera to AChR and myasthenic sera, and for the passive transfer of EAMG.

Characterisation of anti-AChR antibodies

Using the three approaches outlined above much information has accrued about the nature of anti-AChR antibodies. For ease of discussion these characteristics are considered separately for antibodies in the animal model, EAMG, and those in myasthenia gravis itself.

EAMG

When rats were inoculated with eel AChR, antibodies to both eel receptor and rat muscle receptor could be detected in the serum of the animals as early as 3 days after immunisation. By day 10 more than 50% of the antibody population was IgG, with the rest being IgM. Anti-receptor antibodies in rabbits immunised with eel AChR are also IgG (Penn et al., 1976).

In rabbits with chronic EAMG the time course of antibody production correlates with that of disease activity. The intensity of disease appears to have a closer correlation with the titre of antibody to the immunogen than that of antibody directed specifically against the host receptor (Ueno et al., 1980), strongly suggesting that cross-reaction of antibody to conserved antigenic sites, rather than an induced autoimmune reaction to host receptor, plays the major role in the development of EAMG.

In chronic EAMG anti-receptor antibodies are in large excess over muscle AChR, with a large fraction of the muscle receptors having bound antibody, as demonstrated by both direct extraction of antibody-

receptor complexes from muscle (Lindstrom et al., 1976a,b; Lindstrom and Lambert, 1978), and by ultrastructural localisation of bound antibody by staining with peroxidase-conjugated protein A (Sahashi et al., 1978). These bound antibodies are able to bind complement, leading to focal lysis of the post-synaptic membrane (Engel et al., 1977; Sahashi et al., 1978). Anti-AChR antibodies are relatively species specific, with only a small percentage of antibodies to eel or Torpedo AChR showing binding to skeletal muscle receptor from several species (Lindstrom, 1978). This interspecies cross-reaction seems to be dependent on conformationally-dependent antigenic determinants which are not present after receptor denaturation (Lindstrom et al., 1979a).

Antisera to electric fish AChR raised in experimental animals may have a direct effect on AChR function. Greater than 80% blockage of the depolarising response of eel electric organ to carbamycholine can be achieved by preincubation of the organ with anti-receptor antibody (Patrick et al., 1973; Lindstrom et al., 1977). Electrophysiological studies of AChR 'noise' in rat muscle cultures exposed to anti-AChR antibodies show a decrease in the mean open time of 23% (Heinemann et al., 1977). Similar results have been observed with human muscle cells in culture (Bevan et al., 1978). Anti-AChR sera, IgG and Fab fragments have been shown to inhibit ^{125}I -α-BGT binding to both detergent-solubilised and membrane-bound AChR (Penn et al., 1976; Zurn and Fulpius, 1977; Aharonov et al., 1977; Karlin et al., 1978; Claudio and Raftery, 1980; Desouki et al., 1981). Zurn and Fulpius (1977) showed that the titre of antibody which blocks α-BGT binding increased more strongly than antibody directed against other sites just prior to the appearance of paralysis in inoculated animals.

Gomez and Richman (1983) found that 3 mAbs binding at or near the cholinergic binding site had a unique ability to induce an acute neuromuscular disease in chicks, resembling myasthenia gravis, suggesting that the mechanism of immunopharmacological blockade of AChR function is capable of playing a significant role in the pathogenesis of myasthenia. IgG from rabbits and sheep inoculated with Torpedo AChR also inhibit the AChR-induced $^{22}Na^{+}$ - flux in Torpedo electric organ membranes, but do not directly block histrionicotoxin binding to the receptor ion-channel (Desouki et al., 1981).

Anti-receptor antibodies and $F(ab')_2$ fragments have been shown to increase the rate of degradation of AChR from both muscle cells in culture, and of innervated muscle in organ culture (Prives et al., 1979; Lindstrom and Einarson, 1979; Merlie et al., 1979). Corresponding Fab fragments were without effect (Prives et al., 1979), unless a bivalent second antibody was also added (Lindstrom and Einarson, 1979). This phenomenon resembles the antibody-induced degradation of some lymphocyte surface antigens (Old et al., 1968; Cohen and Liang, 1976) and is known as antigenic modulation. It is inhibited by low temperature, and by inhibitors of energy metabolism (Heinemann et al., 1978) and is probably, though not necessarily, dependent on cross-linkage of AChR by the antibody (for review see Patrick and Berman, 1980).

Myasthenia Gravis

Sera from myasthenic patients contain a heterogeneous population of anti-AChR antibodies directed at several antigenic determinants. In most patients anti-receptor antibodies are of the IgG class, with IgA or IgM also being produced in a few (<5%) patients (Tindall, 1981). Studies on the subclass distribution of anti-AChR antibodies have

produced differing results, with a predominance in the IgG 1 or 3 subclasses in one report (Lefvert, 1981) and IgG 1 and 2 subclasses in another (Newsom-Davis and Vincent, 1982).

Four distinct reactivities with AChR have been ascribed to myasthenic antibodies.

1. Antibodies causing a direct immunopharmacological blockade of AChR function. Reductions of α- BGT binding and acetylcholine sensitivity of receptor by myasthenic sera, IgG and $F(ab')_2$ fragments have been demonstrated (Mittag et al., 1976, 1978; Lefvert and Bergstrom, 1977; Harvey et al., 1978; Dwyer et al., 1979; Fulpius et al., 1980; Lefvert et al., 1981). Fulpius et al. (1980) found the α- BGT blocking activity in IgG subclasses 1 and 3, or in some cases exclusively in subclass 3. The presence of antibodies which block toxin binding has been reported to correlate with disease severity, being present only in patients with stage IIB, III or IV disease according to the Osserman classification (Lefvert et al., 1981). The existence of anti-α-BGT site antibody is not common to all myasthenia gravis patients. In a study of 12 patients Whiting et al. (1983) found that anti-toxin site antibodies varied from 0 - 33% of the total anti-AChR antibody population.
2. Antibodies with determinants present exclusively on extra-junctional AChR (Weinberg and Hall, 1979; Dwyer et al., 1981b).
3. Antibodies interacting with the carbohydrate moiety of the AChR (Mittag et al., 1976, 1978, 1981a,b; Dwyer et al., 1981b).

4. Antibodies reacting with determinants outside of the ligand binding site, or carbohydrate groups.

Lefvert (1982) found that anti-AChR antibodies reacted better with a detergent extracted receptor from denervated or myasthenic human muscle than that of normal muscle. A few patients with moderate to severe clinical symptoms possessed antibodies which would react only with myasthenic muscle, and the antibody population of one patient would react only with the patient's own muscle AChR. Vincent and Newsom-Davis (1982) also found anti-'normal' AChR antibody titres to be generally lower than the corresponding anti-'denervated' AChR antibody titre. In contrast Lotwick et al. (1983) found essentially no difference between anti-'normal adult muscle' AChR antibody titre, and the titre against foetal muscle AChR.

Despite the heterogeneity of antibody specificities in myasthenia gravis sera, the serum of each patient seems to be dominated by one antibody type. Thus Bray and Drachman (1982) found that Scatchard analysis of the binding of 15 different myasthenic sera to AChR all showed linearity. The pattern of anti-receptor antibody reactivity to the AChR from different sources is also relatively constant over extended times for any individual patient (Harrison et al., 1981) which is suggestive of an initial limited exposure of the AChR antigen to the immune system.

Myasthenic sera and antibodies are able to produce antigenic modulation (Appel et al., 1977; Heinemann et al., 1977; Drachman et al., 1978, 1982), complement-mediated focal lysis of the neuromuscular junction (Engel et al., 1981; Rash et al., 1981), and specific myolysis of muscle in culture (Liveson et al., 1976). The titre of antibodies able to cause modulation in myasthenic sera was

found to correlate reasonably well with severity of the disease (Drachman et al., 1982).

Antigenicity of the AChR

The classic studies of Atassi (1975, 1978) made it clear that not all parts of a protein are antigenic or immunogenic. A complex multisubunit molecule such as the AChR would be expected to possess a number of antigenic determinants. Lindstrom et al. (1981) predicted a figure of about 10 such determinants, and more recently Gullick and Lindstrom (1982) claimed to have defined a minimum of 28 antigenic determinants of purified Torpedo AChR, although not all of the antigenic determinants were myasthenogenic, and may thus be considered less functionally important.

Although anti-AChR antibodies can block α- toxin or acetylcholine binding to the receptor there is still uncertainty as to whether this is due to a steric hindrance or to direct blockade of the ligand binding site. Fulpius et al. (1980) found antibodies of the IgG 3 subclass in myasthenic sera whose interaction with the AChR was both mutually exclusive with α- BGT, and competitive with low concentrations of some nicotinic, but not muscarinic, ligands. Despite this finding, the overall balance of evidence suggests that the binding site itself is not a major antigenic determinant on the AChR. Thus antibody-receptor complexes extracted from muscle of rats with EAMG were still capable of binding α- BGT (Lennon, 1976). The presence of a free α- BGT binding site on the receptor is also not a requirement for the induction of EAMG (Fulpius et al., 1978).

Antibodies which inhibit Concanavalin A binding to the AChR may do so by steric hindrance rather than by direct binding to the carbo-

hydrate moiety of the receptor, since glycosidase treatment resulting in extensive loss of carbohydrate from Torpedo AChR had no effect on its antigenicity (Weinberg and Hall, 1979; Wonnacott et al., 1980a). In contrast to this Dwyer et al. (1981a) found a greater inhibition of α- BGT binding to extrajunctional AChR than to junctional receptor by some myasthenic sera, which was abolished by treatment of the extrajunctional receptor with glycosidase.

Atassi defined two types of antigenic determinant. "Continuous" determinants are those in which amino acid residues forming the determinant are adjacent. "Discontinuous" determinants, on the other hand, are formed by amino acids from different parts of the sequence brought into close proximity due to folding back of the polypeptide, or by two adjacent polypeptide chains. Antibodies to continuous determinants can bind to the denatured protein chain, while antibodies to discontinuous determinants can bind only to the intact and correctly folded chain. An irreversibly denatured AChR (reduced, carboxymethylated receptor) reacted with only a portion of the antibody population in anti-AChR sera indicating the presence of conformationally-dependent determinants on the intact AChR (Bartfield and Fuchs, 1977). This denatured receptor was unable to induce EAMG in experimental animals, suggesting that discontinuous, conformational determinants are primarily responsible for the myasthenogenicity of the AChR. SDS-denatured AChR was similarly immunogenic (although significantly less so than native receptor) but not myasthenogenic in experimental animals (Lindstrom et al., 1976c).

Each of the subunits comprising the AChR is myasthenogenic (Lindstrom, 1978). Thus, although they are much less immunogenic than native receptor, immunisation with any one of the four polypeptide

chains produces EAMG, with the α- and δ - chains being most effective. Antibodies raised against the polypeptide chains are directed at determinants which normally account for little of the immunogenicity of native AChR, since antibodies to native receptor showed little reaction with the individual polypeptide chains, whereas antibodies to the subunits react quite well with native AChR (Lindstrom et al., 1979b).

Fuchs and coworkers have studied the effects of a number of chemical modifications of the AChR on its antigenicity. Trypsinisation of the receptor led to isolation of a small (27000 dalton) polypeptide which appeared to come from the α- subunit, and bound cholinergic ligands. This trypsinised receptor fragment was able to induce EAMG in rabbits, and thus localised at least one antigenic determinant to a site on the α- subunit, relatively close to the α- toxin binding site (Bartfield and Fuchs, 1979). polyalanylated AChR (Tarrab-Hazdai et al., 1980) bound cholinergic ligands, but was not myasthenogenic, adding further weight to the argument that the ligand binding site is not an important antigenic determinant in EAMG. Treatment of AChR with tetranitromethane specifically modifies tyrosine residues (Sokolovsky et al., 1966). Such a nitrated receptor was devoid of myasthenogenic activity (Fuchs et al., 1981), suggesting that at least some tyrosine residues in the AChR are crucial to its immunogenicity.

In recent years more detailed studies of AChR antigenicity have been made by using mAbs raised against the receptor. Such monospecific antibodies have been elicited by several laboratories (Dwyer et al., 1981b; Gomez et al., 1979; James et al., 1980; Lennon and Lambert, 1980; Mochly-Rosen et al., 1979; Tzartos and Lindstrom, 1980) and are ideal probes to define both the structure of the AChR, and the

specificities of antibodies in myasthenic sera. The most extensive study to date is that of Lindstrom and coworkers who have raised a large library of mAbs aginst Torpedo AChR and have mapped their antigenic specificities on the receptor. A minimum of 28 determinants have been elucidated by this mapping technique (Gullick and Lindstrom, 1982), which are present on all four receptor subunits. About half of the mAbs raised by these workers reacted detectably with denatured subunits or characteristic peptide fragments of the subunit (Tzartos and Lindstrom, 1980; Gullick et al., 1981). Tzartos and Lindstrom (1980) also detected a "main immunogenic region" (MIR) on the α-subunit composed of at least two adjacent antigenic determinants. At least 50% of the antibodies in an antiserum to native AChR were directed at this region. A comparable determinant was also present on the AChR from human muscle (Tzartos and Lindstrom, 1981). The determinants forming the MIR are conformationally dependent, and situated on the extracellular surface of the AChR in situ. The region is included in a peptide fragment containing the α- toxin binding site, but is not the binding site itself (Lindstrom et al., 1981). Competition experiments between myasthenic sera and mAbs directed at the MIR showed that this region is also highly immunogenic in myasthenia patients. Regions on the β- and γ - subunits were also found to be substantially immunogenic (Tzartos et al., 1982).

Finally, several groups have found that extrajunctional rat AChR contained additional antigenic determinants not present on junctional receptor, and that myasthenic sera reacted better with this extrajunctional AChR (Weinberg and Hall, 1979; Dwyer et al., 1981a; Harrison et al., 1981). This finding has yet to be reproducibly confirmed for human AChR (Lotwick et al., 1983) but has important implications for the aetiology of myasthenia gravis.

Antibody and cell-mediated cytotoxicity in myasthenia gravis

It has been found that sera from myasthenic patients have haemolytic complement activities which range widely outside the values for normal subjects (Nastuk et al., 1960). The C3 and C9 components of the complement cascade have been demonstrated at the ultra-structural level in fresh - frozen sections of myasthenic end-plates, with a localisation identical to that of IgG (Engel et al., 1981). The loss of end-plate function is correlated with at least two phases of immunologically mediated destruction. In the first phase, anti-AChR IgG and complement factors Clq and C3 bind to the crests of the junctional folds. In the second, or lytic, phase the crests of the junctional folds are progressively destroyed by IgG - activated complement factors (C5b - C9) resulting in the extrusion of perforated vesicular debris, and extensive lytic damage to the remaining sub-synaptic membrane (Rash et al., 1981). In EAMG complement activation is required for a decrement of compound muscle action potential, and a mononuclear inflammatory response to occur at the motor-end plate following passive transfer of anti-AChR antibodies (Lennon et al., 1978). The activation of complement occurs via the classical pathway (C1→ C2→ C4→ C3→ C5→ 9), rather than the alternative pathway in which the requirements for C1, C2 and C4 are bypassed (Lennon and Lambert, 1981).

Studies of muscle biopsies from myasthenic patients reveal a number of abnormalities including denervation, type II fibre atrophy, lymphocyte infiltration, and fibre necrosis with phagocytosis (Werneck, 1982). Cytolytic effects of peripheral and thymic lymphocytes (Kott and Rule, 1973) and sera (Liveson et al., 1976) from myasthenic patients have also been reported. It is not known whether the myolytic effects of sera are caused by anti-AChR antibodies or by

anti-striational muscle antibodies.

The mechanism of the autoimmune response in EAMG and myasthenia gravis

The development of EAMG is thought to follow the mechanism outlined below. The injection of heterologous AChR possessing cross-reactivity with self AChR induces breakdown of immunological tolerance to the latter receptor, and the animals then start to produce "auto-antibodies" (Patrick and Lindstrom, 1973; Lennon, 1976; Aharanov et al., 1977). Such a break in immunological tolerance has been clearly demonstrated in mice, where 40% of mice immunised with Torpedo AChR possessed a small amount of antibody reactive with antigenic determinants present on mouse AChR, but not present on the Torpedo receptor. Lindstrom (1977, 1979) suggested that the immune response in EAMG is caused by heterologous AChR, but is sustained by auto-stimulation with self-receptors from muscle destroyed during the initial immune response. Berman et al. (1981) found that the susceptibility of mice to EAMG is a heritable trait, and that regions of the mouse genome which regulate immune responsiveness (MHC and IgC_H) can profoundly affect the probability with which a mouse immunised with AChR can be expected to become paralysed.

The initiating factor of the autoimmune response in myasthenia gravis is not known. The evidence to date suggests that the auto-antigen is the AChR. Because sera from myasthenic patients may react better with extrajunctional than junctional receptor, the initiating antigen may be other than normal junctional AChR. It is not known whether the antibody present in myasthenic serum is directed solely at antigenic determinants on the extracellular surface of the AChR, or whether intracellular determinants are also important. If intra-cellular determinants are involved it would suggest that the initiating

factor in myasthenia may be the shedding of receptor from the post-synaptic membrane, creating an effectively foreign antigen.

The thymus has long been thought to be involved in the pathogenesis of myasthenia gravis. Thymic abnormalities such as hyperplasia, increased numbers of B - cells and decreased numbers of T - cells may be be found in the myasthenic thymus (Castleman, 1966; Abdou et al., 1974; Levine and Rice, 1977), and thymectomy is known to benefit many patients (Simpson, 1958; Papatestas et al., 1971). The thymus may be involved in the pathogenesis of myasthenia, firstly by serving as a potential site of antigen, which plays a role in the breakage of tolerance toward self - AChR, and secondly as a site of anti - AChR antibody synthesis. In support of the latter is the presence of anti - AChR antibody in the myasthenic thymus (Mittag et al., 1976) and the spontaneous synthesis of anti - AChR antibody by thymic cells from myasthenic patients in culture (Vincent et al., 1978). Thymic epithelial cells appear to bear AChR (Engel et al., 1977), and myoid (muscle-like) cells grown out in explant culture from rat and human thymus express AChR (Kao and Drachman, 1977). In addition, immunofluorescence studies using EAMG sera have shown binding to about 80% of mouse thymocytes, suggesting the presence of an AChR-like antigen on the surface of these cells.

Altered immunoregulatory functions may be operative in myasthenia gravis. Studies of suppressor T - cell activity in myasthenia have been extensive, but have yielded conflicting results. Suppressor T - cell activity as measured by Concanavalin A and phytohaemagglutinin has been reported to be diminished by some authors, while others have found no suppressor differences between controls and myasthenic patients (Koethe et al., 1981). Percentage estimates of T - cell

subsets using mAbs have also yielded equivocal results. OKT8 - positive cells (representing suppressor/cytotoxic cells) have been variously reported as decreased, normal or increased. Levels of T - cells bearing the Fc receptor for γ - immunoglobulin (also thought to represent suppressor cells) were also found to be elevated, depressed or normal in a stable myasthenic population (for review see Seybold, 1983). Newsom-Davis et al. (1981) have shown that thymus cells can selectively enhance anti - AChR antibody production by autologous peripheral blood lymphocytes. Other immunological abnormalities in myasthenia gravis include the presence of anti - T cell antibodies, and elevated levels of thymosin α_1 (a polypeptide which is a potent immunopotentiating agent that can increase cell growth, influence antibody response, or affect maturation and differentiation of T - lymphocytes) in epithelial cells of the myasthenic thymus. An additional argument for an immune dysfunction is that more than one autoimmune disorder often occurs concurrently in the same patient (Waldmann et al., 1978).

It is possible that myasthenia gravis may be the result of a genetic predisposition to an altered immune response. Thus in some patients there is a significantly increased frequency of the major histocompatability complex gene products HLA - B8 and DRw3 (Safwenberg et al., 1978) and HLA - B12 in others (Yoshida et al., 1977). The high incidence of HLA - B8 is also found in other autoimmune disorders, and suggests that these antigens are responsible for an increased susceptibility to autoimmune disorders rather than the initiation of an immune response specifically to the AChR (Lindstrom, 1979).

The study of myasthenia gravis has far-reaching consequences in

that, as an established autoimmune disorder where the auto-antigen is known, it may be useful in gaining a deeper understanding of other autoimmune disorders. In this study the antigenic nature of purified Torpedo AChR has been examined with particular reference to the role of the α- toxin binding site, and the carbohydrate moiety of the receptor in its antigenicity. Because the AChR is an integral membrane protein, a considerable portion of its structure may be hidden from humoral circulation. Therefore, the cross-reaction of anti-Torpedo AChR antibodies with the AChR of muscle cells in culture has also been studied. Such an approach also has the advantage of detecting only cross-reacting and presumably highly conserved determinants which might be expected to have a functional significance in the pathogenicity of EAMG.

The contribution of antibody-mediated muscle cell damage in the pathology of myasthenia gravis has been relatively little studied. The second aim of this study has, therefore, been to make an assessment of the extent and nature of the myolytic effects of myasthenic sera.

MATERIALS and METHODS

Preparation of chick embryo extract

10 - 12 day old chick embryos (Maurice Millard Ltd, Freshford, Bath) were aseptically squeezed through a 50 ml syringe, and diluted with an equal volume of sterile Earle's balanced salt solution (BSS). After standing at 22°C for 2 h the suspension was centrifuged in sterile tubes at 30,000g for a further 2 h. The supernatant was removed aseptically, and aliquots (5 ml) were stored in sterile glass universal bottles at -20°C.

Preparation of rat-tail collagen

Collagen was prepared from rat tails essentially according to the procedure of Ehrmann and Gey (1956). A single rat tail was washed with 70% alcohol for 10 min. The skin was stripped off and the tendons aseptically dissected out using sterile instruments. The tendons were placed in sterile 0.1% (v/v) acetic acid (50 ml) for 24 h at 4°C. At the end of this period the residue of the tendons was removed by centrifugation (20,000 g) at 4°C for 90 min. in sterile, capped centrifuge tubes. The supernatant collagen solution was transferred to a sterile media bottle and stored at 4°C until required.

Collagen coating of tissue culture dishes

Collagen solution (50 µl) and sterile 6% (w/v) sodium chloride solution (20 µl) were placed separately in the bottom of a 35 mm diameter tissue culture dish (Gibco Nunc Ltd, Paisley, Scotland), and then mixed and spread evenly over the surface using a sterile L - shaped glass rod. The collagen matrix was precipitated by leaving the dishes for at least 48 h at 37°C.

The preparation of myotube cultures from chick embryos

Myotube cultures were produced from the hind limbs of 10 - 11 day old chick embryos by a procedure based on the methods described by Harvey and Dryden (1974). The dissociation procedure is shown schematically in Figure 7.

10 - 11 day fertilised eggs were washed with 70% (v/v) alcohol and placed in a laminar flow cabinet. Each egg in turn was broken into a sterile glass Petri dish. The embryo was picked out using sterile forceps, and placed into a sterile glass dish containing BSS (approximately 100 ml). One embryo was then placed into another sterile glass Petri dish on the stage of a Swift zoom dissecting microscope set at approximately 7 - 10 times magnification. One leg was held away from the body using a pair of sprung-loaded irridectomy scissors (Johan Weiss Ltd, London). The leg was then cut along the arc line joining the thigh to the trunk, and carefully removed to a sterile glass Petri dish containing BSS. The embryo was turned, and the second leg similarly removed. The procedure was repeated for each embryo. When all the legs were removed, one leg was transferred back to the Petri dish on the microscope stage and the magnification increased to 10 - 20 times. Remaining skin was removed from the leg, and muscle was teased away from bone and tendons using two pairs of sterile Dumont number 5 watchmakers forceps. This procedure was repeated for each leg. The muscle mass obtained was kept moist with BSS during the dissection procedure. The muscle mass was finely minced with sterile irridectomy scissors, and transferred into a solution (10 ml) of sterile trypsin (0.1% (w/v) in calcium and magnesium-free BSS: formulation shown in Table 5) using a sterile 10 ml glass pipette. The suspension was vigorously agitated with a whirlimixer, and incubated for 30 min at 37^{o}C, whirlimixing once

FIGURE 7. Preparation of a single-cell suspension from chick embryo hind limb muscle.

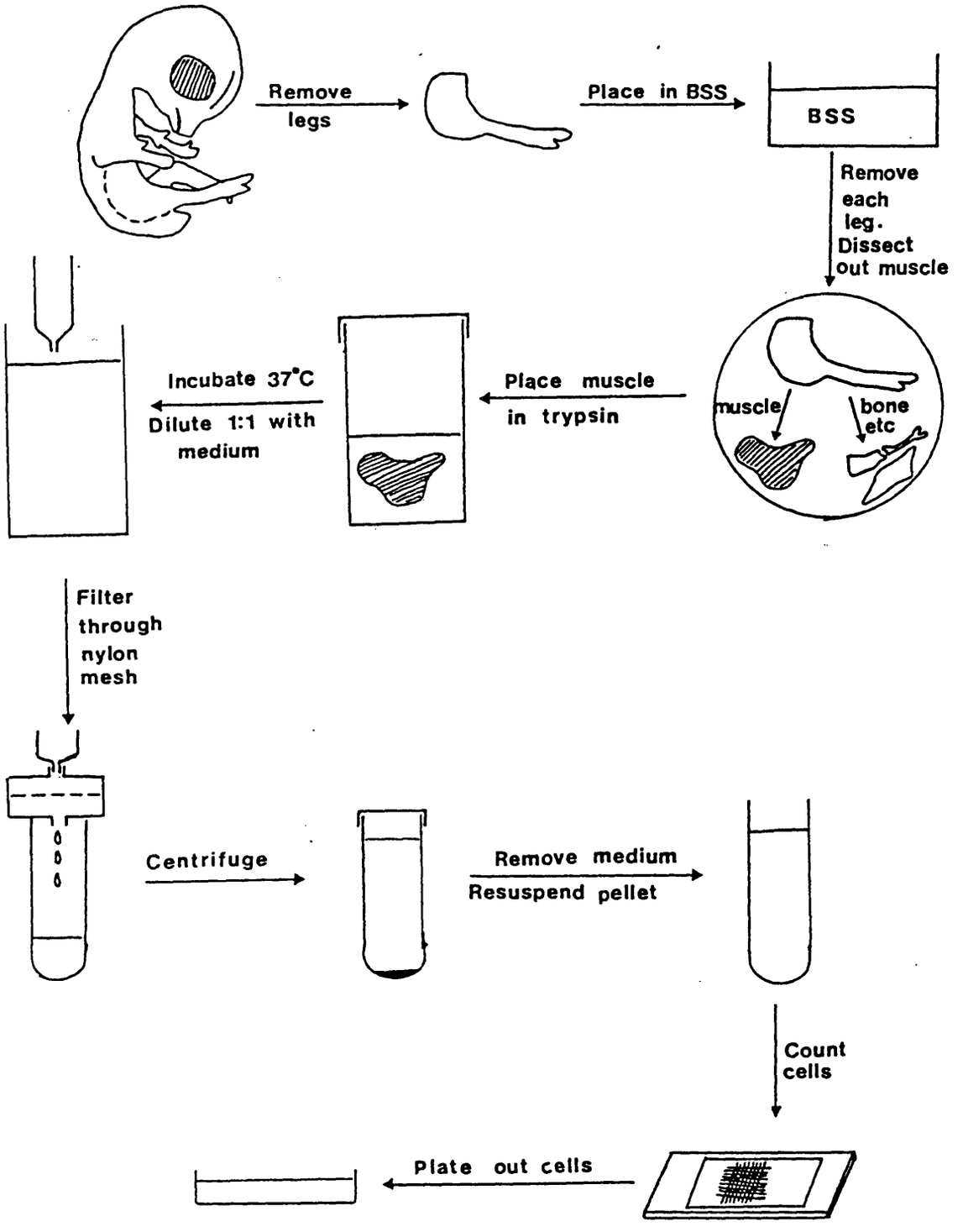


TABLE 5. Composition of physiological salt solutions used in the preparation and assay of muscle cultures.

Calcium/magnesium-free Earles balanced salt solution.

Component	g/l
NaCl	69
KCl	4
NaH_2PO_4	1.4
$NaHCO_3$	16.8
Na_2SO_4	2.6
Glucose	10
Phenol red	3ml/l of 0.5%w/v

Made up to 1000ml with double distilled water.

Sterilised by filtration to 0.22μm

Dulbecco's phosphate buffered saline.

Component	g/l
NaCl	8
Na_2HPO_4	1.15
KH_2PO_4	0.2
KCl	0.2
$CaCl_2.2H_2O$	0.132
$MgCl_2.6H_2O$	0.1

Made up to 1000ml with double distilled water.

halfway through the incubation, and again at the end of the incubation. Following this, an equal volume of cold (4°C) minimal Eagle's medium, containing 5% (v/v) chick embryo extract and 15% (v/v) heat inactivated horse serum (MEM(i)) was added to the muscle cell suspension, and the suspension triturated with a 10 ml pipette to remove any lumps. The suspension was filtered through two layers of sterile 50 μm mesh nylon cloth (Simon Textiles Ltd, Cheshire) into two 10 ml sterile centrifuge tubes, and centrifuged at 80 g for 15 min at room temperature. The resulting supernatant was discarded, and the cell pellet resuspended in MEM (i) (10 ml) by gentle trituration with a Pasteur pipette. The cell suspension, comprising mostly myoblasts, with a few fibroblasts and erythrocytes, was counted in a haemocytometer. Erythrocytes were clearly identifiable and were excluded from the count. The cell suspension was diluted with MEM (i) to give a suspension containing 5×10^5 cells/ml, and plated out into collagen-coated 35 mm tissue culture dishes at a density of 1×10^6 cells/dish. The dishes were placed in an incubator at 37°C under an atmosphere of 5% CO_2 in air.

After approximately 52 h incubation (when myoblast fusion was essentially complete) the medium was replaced with MEM (ii) (formulation as for MEM (i) but containing only 2.5% (v/v) chick embryo extract) containing 10^{-5} M cytosine arabinoside (2 ml/dish) and the dishes re-incubated. After a further 60 h incubation, the medium was replaced with MEM (ii) without cytosine arabinoside (2 ml/dish) and the dishes incubated as previously. Myotube cultures were used for further experiments at 6 - 9 days after initial plating.

Preparation of human foetal myotube cultures

Limbs were obtained from suction terminations of 8 - 14 week

old foetuses, by sieving the aspirate through a sterilised metal kitchen sieve under running tap water. The limbs were transported dry to the laboratory, and then immediately placed into sterile culture medium.

Disaggregation of tissue: Muscle was teased away from the limbs, after removal of the skin, using Dumont number 5 watchmakers forceps. The muscle mass obtained was finely minced with sprung-loaded irridectomy scissors.

The minced muscle was disaggregated by either mechanical or enzymatic means.

(i) Mechanical disaggregation: Minced muscle was placed in sterile PBS (5 ml) and agitated for 1 min on a vortex-mixer, then vigorously triturated with a Pasteur pipette. Large lumps were allowed to settle, and the cell suspension was filtered through two layers of sterile 50 μm mesh nylon cloth. The resulting cell suspension was passed through a syringe fitted with a 19 - gauge needle to disaggregate any remaining cell clusters.

(ii) Enzymatic disaggregation: The minced muscle was placed in PBS (5 ml) containing either trypsin (0.1% (w/v)), or collagenase (type II; 0.5% (w/v)) and deoxyribonuclease (0.002% (w/v)), and incubated at 37°C for 30 or 60 min with a brief mix on a vortex mixer every 15 min. Following this incubation, cold (4°C) culture medium (5 ml) was added, and the suspension was triturated with a Pasteur pipette. The suspension was filtered through two layers of 50 μm mesh nylon cloth and then passed through a syringe fitted with a 19 - gauge needle to remove any remaining cell aggregates.

To compare the disaggregation methods, the minced muscle mass

from a single foetus was divided into roughly equal portions, and the portions were placed into the disaggregation solutions. The weight of muscle was determined, and the disaggregation procedures followed.

Plating out and culture of muscle cell suspensions: Disaggregated cell suspensions were centrifuged (80 g, 10 min). The medium was decanted, and the pellet was resuspended in culture medium (20 ml). The suspension was re-centrifuged, and the cell pellet was resuspended in fresh culture medium. The viability of the resulting cell suspension was determined by mixing a sample (200 µl) 1:1 with 0.2% trypan blue (Phillips, 1973), and the viable nucleated cell count calculated. The cell suspension (2 ml) was plated out into collagen-coated 35 mm diameter culture dishes at a viable cell concentration of 10^5 - 10^6 cells/dish. Some cell suspensions were "preplated" into 75 cm^2 surface area tissue culture flasks before plating out into culture dishes. To do this, the cell suspension (1 - 1.5 x 10^6 cells/ml; total volume 10 ml) was placed into a 75 cm^2 flask for 40 min at 37°C. Following this, unattached cells were removed by decanting off the medium. This cell suspension was then plated out into culture dishes.

The medium in the culture dishes was changed every 2 - 3 days. Cytotoxic drugs were added at days 3 - 5 in culture, and incubated with the cell monolayers for 24 - 60 h.

Cell staining

Muscle cell monolayers were stained with haematoxylin and eosin by the following procedure. Medium was decanted from culture dishes, and the cell monolayer was washed once with PBS (2 ml). The cell monolayer was fixed with industrial alcohol for 2 min. Harris haematoxylin (approximately 1.5 ml) was added and the cells were

stained for a further 2 min. The stain was removed, and the cells were rapidly washed with running tap water. Eosin (1% (w/v) eosin Y in distilled water) was added, and the cell monolayer was counter-stained for 2 min. Excess stain was removed by rinsing with tap water. The stained monolayers were mounted with Apathy aqueous mounting medium, and photographed on a Zeiss Ergoval bright-field microscope using Kodak Ekta 50 or 160 ASA colour film.

Preparation of sheep sera

For preparation of normal sheep serum, blood was collected into one litre centrifuge bottles after cutting the jugular vein of stunned sheep (this procedure was kindly performed by staff at the Bath abattoire). The blood was clotted at 37°C for 1 h, and then centrifuged (1000 g) for 30 min at room temperature. The serum was removed by aspiration, and aliquots (20 ml) stored frozen at -20°C.

To prepare sheep anti-(Torpedo AChR) antiserum, purified AChR from Torpedo marmorata (80 µg) in Complete Freund's adjuvant was injected intramuscularly, followed by a similar injection 3 weeks later. Five days after the second injection the sheep became paralysed and was killed. Blood was collected and serum obtained as described for normal sheep serum.

Preparation of IgG from sheep serum

IgG was prepared from sheep anti-(Torpedo AChR) antiserum, or normal sheep serum by ammonium sulphate precipitation, and ion-exchange chromatography following the method of Stevenson and Dorrington (1970). Serum was made to 45% saturation with ammonium sulphate, by the addition of saturated ammonium sulphate solution (60 ml per 100 ml of serum). The solution was stirred gently for 30 min , and

the resulting precipitate collected by centrifugation (500 g) for 15 min at 4°C. The precipitate was redissolved in 0.2 M Tris buffer, pH 8.0 to the original serum volume, and re-precipitated by the addition of saturated ammonium sulphate (60 ml per 100 ml redissolved precipitate). This procedure was repeated until a white precipitate was obtained. The final precipitate was dissolved in 0.03 M sodium phosphate buffer, pH 7.3, and the solution dialysed against the same buffer (2 l) at 4°C overnight. The dialysate was applied to a column (10 x 2 cm) of DEAE - cellulose (DE52, microgranular form) pre-equilibrated with dialysis buffer. The column was eluted using the same buffer. IgG passed straight through the column, while other immunoglobulins remained bound to the ion-exchange resin. Elution of IgG from the column was monitored by absorbance at 280 nm of the eluate using an LKB Uvicord I ultraviolet monitor, and the IgG collected as a single fraction. The IgG peak fraction was concentrated on a millipore ultrafiltration unit to a final concentration of 10 - 20 mg/ml, and stored at -20°C for further use.

Preparation of $F(ab')_2$ fragments from sheep IgG

$F(ab')_2$ fragments were prepared by peptic digestion of IgG by a modification of the method of Hudson and Hay (1976). IgG (20 mg/ml in 0.03 M sodium phosphate buffer, pH 7.3) was dialysed for 3 h against 0.1 M sodium acetate (2 l), and the pH of the resulting dialysate adjusted to 4.5. Lyophilised pepsin (2 mg per 100 mg IgG) was added to the dialysate, and the mixture incubated at 37°C for 24 h. The solution was then centrifuged (1000 g) for 15 min at room temperature, and any precipitate discarded. The pH of the supernatant was adjusted to 7.4, and it was then loaded onto a column (100 x 3 cm) of Ultrogel ACA34. The column was eluted with 0.1 M Tris buffer containing 0.15 M NaCl, pH 8.0. Fractions (7 ml) were collected, and the

absorbance at 280 nm of the column eluate was monitored. The fractions corresponding to $F(ab')_2$ were pooled, dialysed against 0.1 M sodium phosphate buffer, pH 7.0, and concentrated to 3 mg/ml. Samples (1 ml) were stored at -20°C for further use. $F(ab')_2$ fragments from normal sheep IgG were termed 'non-immune' $F(ab')_2$, and those from sheep anti-(Torpedo AChR) IgG, 'immune' $F(ab')_2$.

Affinity purification of anti-(Torpedo AChR) $F(ab')_2$ fragments

α-BGT (2 mg) was coupled to cyanogen bromide-activated Sepharose 4 B (20 ml in 0.2 M $NaHCO_3$, pH 9.4) following the method of March et al. (1974). Any unreacted groups were blocked with 1M glycine, pH 9.0, and the beads were washed successively with 0.1 M sodium acetate, pH 4.0 containing 1M NaCl, and 0.1 M sodium borate, pH 8.0 containing 1M NaCl. The toxin-Sepharose was stored in 10 mM phosphate, pH 7.2, containing 0.1% (w/v) NaN_3 at 4°C.

Purified Torpedo AChR was bound to the toxin-Sepharose as follows. AChR (1.5 nmoles α-BGT binding sites) in 10 mM sodium phosphate buffer, pH 7.2, containing 1% (v/v) Triton X100 was added to toxin-Sepharose (2.5 ml; 25 nmoles α-BGT) in a 5 ml glass pipette. The AChR solution was allowed to drain down, and the pipette was then stoppered and left at 4°C overnight. The column was washed successively with 10 mM sodium phosphate buffer, pH 7.0,containing 0.15 M NaCl and 0.1% (v/v) Triton X100 (5 ml), 10 mM sodium phosphate, pH 7.0 containing 0.15 M NaCl alone (5 ml), and 0.2 M ammonium hydroxide (5 ml) to reproduce the $F(ab')_2$ elution conditions. Assay of the effluent for α-BGT binding sites indicated that more than 99% of the AChR was bound. The column was washed and stored in 10 mM sodium phosphate, pH 7.0, containing 0.15 M NaCl, at 4°C.

Immune $F(ab')_2$ fragments (1.5 ml; 3 mg/ml) were applied to the

column and allowed to drain down. The column was stoppered and left for 90 min at room temperature, or overnight at 4°C to allow anti-AChR $F(ab')_2$ fragments to bind. The column was washed with 10 mM sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl until the absorbance of the effluent at 280 nm returned to zero. The immunospecifically bound $F(ab')_2$ fragments were eluted with 0.2 M ammonium hydroxide (Martinez et al., 1977). Fractions showing an increase in absorbance at 280 nm were quickly desalted by passage through Sephadex G50 (2 ml in a Pasteur pipette) pre-equilibrated with 0.1 M sodium phosphate buffer, pH 7.0, and the peak tubes were dialysed against the same buffer at 4°C overnight. The dialysate was concentrated by using a Minicon concentrator to give a final protein concentration of 100 - 200 μg/ml, and was stored at 4°C. It could be re-used with reproducible results several times over a period of a few weeks.

Affinity chromatography was used primarily to give a measure of the percentage of specific anti-Torpedo AChR antibody within the parent immune $F(ab')_2$ preparation (see Results p.138). The resulting affinity purified anti-AChR $F(ab')_2$ fragments were used only to show the purity of the iodinated parent immune $F(ab')_2$ preparation in a competition experiment (Figure 40). For all other experiments in this thesis involving unlabelled or iodinated immune $F(ab')_2$, the whole $F(ab')_2$ population from sheep anti-Torpedo AChR antiserum was used, without further purification of the specific anti-AChR $F(ab')_2$, and it is this unpurified $F(ab')_2$ preparation which is referred throughout as "immune $F(ab')_2$".

Preparation of Fab fragments from sheep IgG

IgG was dialysed overnight at 4°C against 0.1 M sodium phosphate buffer, pH 7.0, containing 10 mM cysteine and 2 mM EDTA (2 l), and the concentration of the dialysate was adjusted to 10 mg/ml. Lyophilised papain (1 mg per 100 mg IgG) was added, and the mixture was incubated at 37°C for 16 h. The digest was dialysed against 10 mM sodium phosphate buffer, pH 8.0 (2 l) overnight at 4°C, and the resulting dialysate applied to a column (10 x 1.5 cm) of DEAE - cellulose (DE 52) pre-equilibrated in dialysis buffer. Fab fragments passed straight through the column, while Fc fragments and any undigested IgG remained bound to the ion-exchange resin. The eluate fractions containing Fab were pooled, dialysed against 0.1 M phosphate buffer, pH 7.0 and concentrated to 3 mg/ml. Aliquots (1 ml) were stored at -20°C. Fab

fragments were designated 'immune' and 'non-immune' as for $F(ab')_2$ fragments.

Preparation of α-toxin affinity beads

α-toxin purified from Naja naja siamensis crude venom (Cooper and Reich, 1972) was coupled to Sepharose 4 B following the method of March et al. (1974). Sepharose 4 B (50 ml packed beads) was washed with 0.1 M NaCl (1000 ml), and distilled water (500 ml). The beads were resuspended in ice cold distilled water to give a final volume of 100 ml, and 2 M Na_2CO_3 (100 ml) was added. The solution was left to stir at 4°C. α-toxin (25 mg) was dissolved in 0.2 M $NaHCO_3$, pH 9.4 (100 ml) and the absorbance at 280 nm measured. Cyanogen bromide (3g) dissolved in acetonitrile (1.5 ml) was added to the Sepharose 4B solution, and stirred for 2 min at 4°C. The mixture was rapidly filtered and washed with ice-cold distilled water (500 ml). The Sepharose 4 B beads were added to the α-toxin solution, and stirred overnight at 4°C. The beads were washed with distilled water (400 ml), resuspended in 2 M glycine, pH 9.0 (200 ml) and stirred overnight at 4°C. The affinity beads were collected by filtration, and washed successively with 0.1 M sodium acetate buffer, pH 4.0 containing 1 M NaCl (150 ml), and 0.1 M borate buffer, pH 8.0 containing 1 M NaCl (150 ml). The process was repeated three times, and the affinity beads were then equilibrated with 10 mM potassium phosphate buffer, pH 7.4 containing 0.1% (v/v) Triton X100. The beads were stored at 4°C in the presence of 0.02% (w/v) NaN_3. After use, the affinity beads were regenerated by washing with 10 mM phosphate buffer, pH 7.4 containing 1 M NaCl (300 ml) followed by buffer alone (500 ml). Over 95% of the α-toxin was bound to the Sepharose 4 B during the coupling process.

Purification of the AChR from Torpedo marmorata

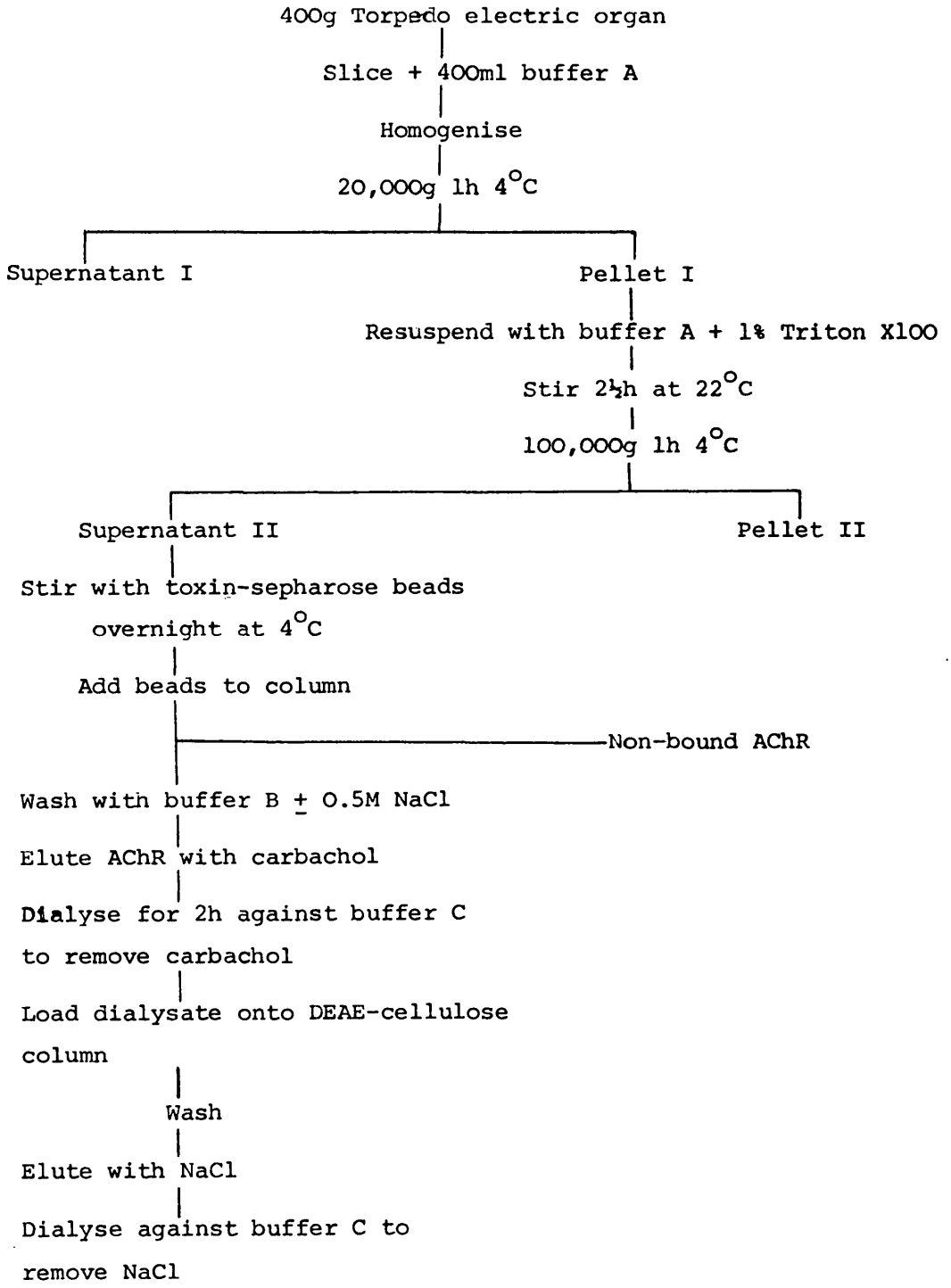
Figure 8 shows the basic procedure for the purification of the AChR from Torpedo marmorata electric organ.

Buffer A contained 10 mM potassium phosphate, 10 mM EDTA, 0.02% (w/v) NaN_3 and 0.1 mM phenyl methyl sulphonyl fluoride (PMSF) at pH 7.4. Homogenisation was performed in a Sorvall Omnimix for 3 periods of 1 min at full speed. The 100,000 g supernatant recovered from the extraction procedure was applied as a batch to the affinity beads and stirred overnight at 4°C. The beads were packed into a column (3 x 9 cm) and washed with buffer B (10 mM phosphate, pH 7.4, 10 mM EDTA, 0.2% (w/v) NaN_3, 0.1% (v/v) Triton X100, 0.1 mM PMSF, containing 1 M NaCl (150 ml), followed by buffer B alone (150 ml). AChR was eluted from the column with 1 M carbamylcholine in buffer B (50 ml) over 3 h at 22°C. Dialysis was carried out against buffer C (2 l; 10 mM phosphate, 0.1% (v/v) Triton X100, 0.2% (w/v) NaN_3, and 0.1 mM PMSF, pH 7.4). Residual carbamylcholine was removed by passage through a column (1 x 1 cm) of DEAE - cellulose (DE 52), and the column was washed with buffer C (2 l). The AChR was eluted with buffer B without PMSF, but containing 0.5 M NaCl. Twenty fractions (1 ml) were collected and analysed for α-BGT binding. The peak fractions were pooled and dialysed against buffer C without PMSF (2 x 2 l). The purified AChR was stored at 4°C.

Preparation of ^{125}I -α-BGT

α-BGT was labelled with ^{125}Iodine (^{125}I) by the method of Urbaniak et al. (1973). Carrier-free $Na^{125}I$ (100mCi/ml) in dilute NaOH solution (10 µl) was added to α-BGT (10 µg) dissolved in 50 mM potassium phosphate buffer, pH 7.5 (20 µl). Chloramine T (0.5% (w/v) in the same buffer; 10 µl) and buffer alone (10 µl) were added. The

FIGURE 8. Preparation of AChR from Torpedo marmorata electric organ.



mixture was stirred at 22°C for 1 min , after which 0.016% (w/v) sodium metabisulphite in buffer (750 µl), and 1% (w/v) potassium iodide in buffer (200 µl) were added to stop the iodination reaction. ^{125}I - α-BGT was separated from free ^{125}I by passage through a Sephadex G25 column (25 x 1 cm) pre-equilibrated with 10 mM potassium phosphate buffer, pH 7.4, containing 1% (w/v) bovine serum albumin, and eluted with the same buffer. Fractions (1 ml) were collected and samples (5 µl) counted in an LKB 1280 Ultrogamma counter. Peak tubes were pooled and the specific activity of the ^{125}I -α-BGT calculated, assuming 100% recovery of protein. The biological activity of the toxin, and the amount of ^{125}I - protein precipitated by 6% (w/v) trichloroacetic acid (TCA) were determined. ^{125}I -α-BGT was stored at 4°C in elution buffer for up to 3 weeks.

Biological activity of ^{125}I -α-BGT

The biological activity of the ^{125}I -α-BGT was determined by measuring the amount of ^{125}I - toxin bound to an excess of purified Torpedo AChR. The assay used was a modification of the method of Schmidt and Raftery (1973), which is detailed below.

Acid precipitability of ^{125}I -α-BGT

The radiolabelled α-BGT was diluted 1 in 100 with 10 mM potassium phosphate buffer, pH 7.4, containing 1% (w/v) bovine serum albumin. Four replicate samples of the diluted toxin (50 µl) were placed on ice, and 12% (w/v) TCA (50 µl) added. After 30 min the precipitate was collected on a Whatman GFC filter, washed with 6% (w/v) cold TCA (5 ml) and counted in an LKB 1280 Ultrogamma counter. Acid-precipitable radioactivity was expressed as a percentage of the total radioactivity added.

Fractionation of ^{125}I -α-BGT

^{125}I -α-BGT was separated into mono- and di-iodinated species by the method of Vogel et al. (1972). Fractions containing ^{125}I - bound to α-BGT after elution from Sephadex G25 were pooled and diluted 1 in 3 with double distilled water. The diluted solution was applied to a column (1 x 1 cm) of CM - Sephadex C 50 pre-equilibrated with 3.3 mM sodium phosphate buffer, pH 7.4 containing bovine serum albumin (2 mg/ml). The column was washed with the same buffer to remove any remaining free iodine, and fractions (1 ml) were collected. The column was eluted with a linear gradient consisting of 40 ml of 3.3 mM phosphate buffer, pH 7.4 containing bovine serum albumin (2 mg/ml), and 40 ml of the same buffer with the addition of 80 mM NaCl. Fractions (1 ml) were collected, and samples (5 µl) of each fraction were counted in an LKB Ultrogamma counter.

For preparative fractionation of ^{125}I -α-BGT, toxin was first labelled at 10 times the concentration of the normal iodination procedure. This was done by increasing the volume of α-BGT, $Na^{125}I$ and chloramine-T by 10-fold, and omitting the buffer addition. The volume and concentration of potassium iodide were increased to 1 ml and 20 mg/ml respectively. The concentration of sodium metabisulphite was increased to 8 mg/ml, and the volume reduced to 200 µl. All other conditions were as described above.

Iodination of $F(ab')_2$ and Fab fragments

Immune and non-immune $F(ab')_2$ and Fab fragments were labelled with ^{125}I essentially according to the method of McConahey and Dixon (1966).

$F(ab')_2$ or Fab (3 mg in 1 ml of 0.1 M phosphate buffer, pH 7.0) were labelled with carrier-free $Na^{125}I$ (100 mCi/ml) in dilute NaOH

solution (10 µl) by the addition of chloramine - T (200 µg/ml in double distilled water; 100 µl). After 5 minutes stirring at 22°C sodium metabisulphite (200 µg/ml in double distilled water; 100 µl) was added to stop the reaction. The iodinated protein was separated from free ^{125}I by passage through a Sephadex G 50 column (25 x 1 cm) pre-equilibrated with 0.1 M sodium phosphate buffer, pH 7.0, containing 1% (w/v) bovine serum albumin, and eluted with the same buffer. Fractions (1 ml) were collected, and samples (5 µl) of each fraction were counted in an LKB 1280 Ultrogamma counter. The peak tubes were pooled, and the specific activity of the ^{125}I - protein calculated, assuming 100% recovery of protein. ^{125}I - $F(ab')_2$ and Fab were stored at 4°C in elution buffer until required.

SDS - polyacrylamide gel electrophoresis

The purity of Torpedo AChR and $F(ab')_2$ and Fab preparations was assessed by polyacrylamide gel electrophoresis under denaturing conditions according to the method of Weber and Osborn (1969). AChR was run under reducing conditions, while Fab and $F(ab')_2$ fragments were run under non-reducing conditions. To prepare samples for electrophoresis, AChR (20 - 50 µg) was boiled for 2 min in a solution containing 3% (w/v) sodium dodecyl sulphate (SDS) and 1% (v/v) 2 - mercaptoethanol in 8 M urea. $F(ab')_2$ and Fab (30 - 60 µg in 10 - 20 µl 0.1 M phosphate buffer, pH 7.0) were mixed with a solution of 3% (w/v) SDS in 8 M urea (80 µl).

Electrophoresis was carried out in 8% acrylamide slab gels. Samples were loaded at 30 mA per gel and run at 100 mA for 2 h at 22°C. Bromophenol blue (0.05%) in 50% glycerol was used as a tracking dye. BDH standard molecular weight markers (14300 - 71500 daltons) were used in determining the molecular weights of stained bands. Gels were

stained with 0.1% (w/v) Coomassie Brilliant Blue R (Sigma Chemical Co. Ltd) in 10% (v/v) acetic acid, 20% (v/v) alcohol. They were destained prior to photography with 10% acetic acid, 20% alcohol, by washing the gel four times for 2 h each.

Protein estimation of myotube cultures

The incubation medium was removed from myotube culture dishes, and each dish was washed three times with Dulbecco's phosphate buffered saline (PBS; 2 ml). 0.1 M NaOH (1 ml) was added to each dish, and the dishes were incubated at room temperature for 10 - 30 min. At the end of this incubation the suspended cells were transferred to a stoppered tube. Each dish was washed with 0.1 M NaOH (1 ml), and the washings were transferred to the appropriate tubes. Cell suspensions were vortex-mixed thoroughly before assaying for protein.

Protein determination was performed by the method of Lowry et al. (1951). Bovine serum albumin was used as the standard protein, and the standard calibration curve is shown in Figure 9A. Cell suspensions were diluted 1 in 5 for protein determination. The protein content of a cell-free plate was taken as a blank in the estimation of total cell protein in myotube cultures.

Protein estimation of $F(ab')_2$ and Fab fragments

Protein estimation of the $F(ab')_2$ and Fab preparations used in this study was performed by the Biuret method (Layne, 1957). Bovine serum albumin was used as the standard protein, and the standard calibration curve is shown in Figure 9B.

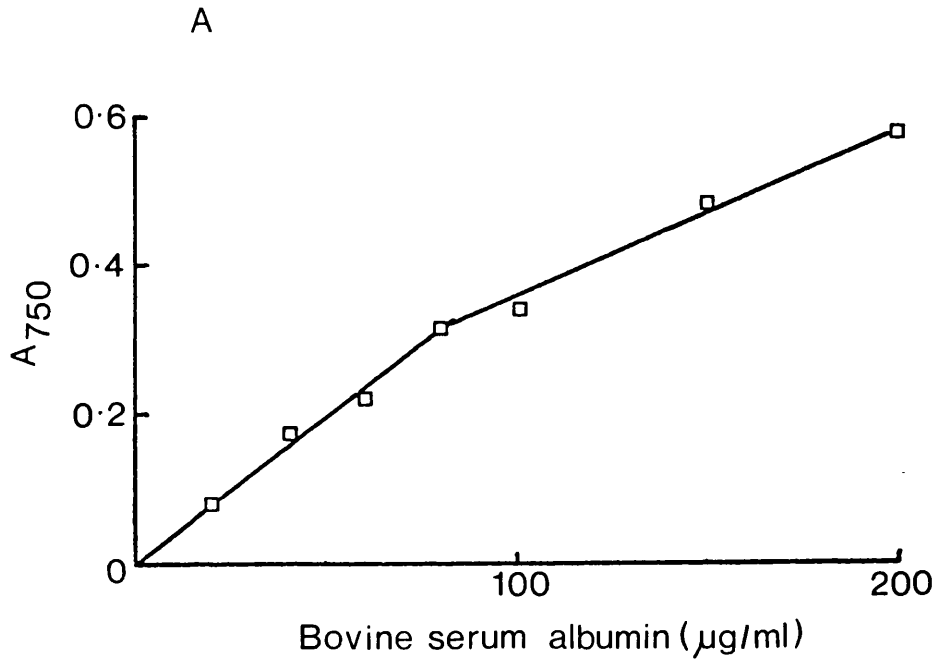
^{125}I -α-BGT binding to Torpedo AChR

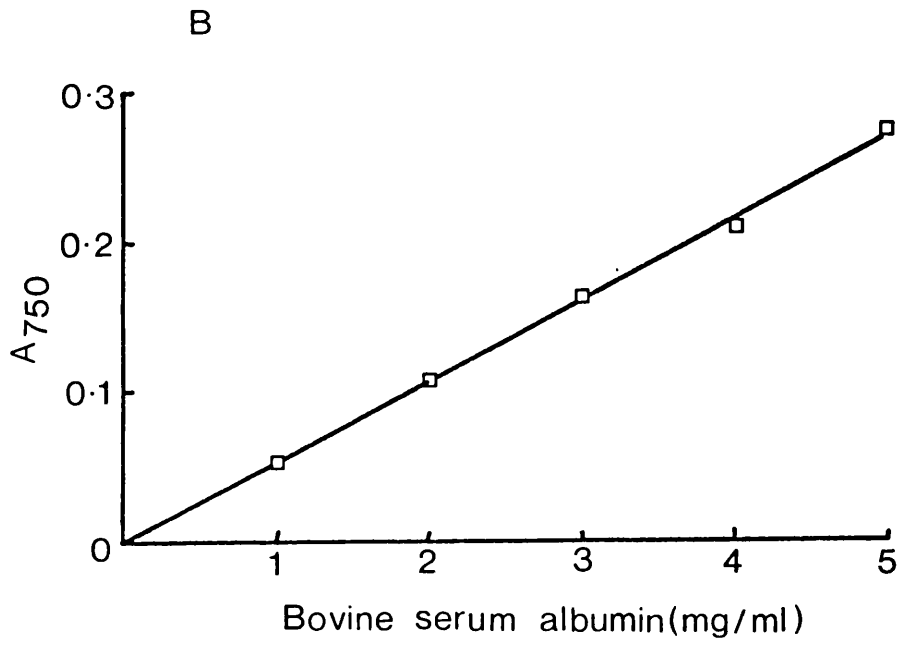
^{125}I -α-BGT binding to purified Torpedo AChR was assayed by a

FIGURE 9. Standard curves for protein estimation.

A : Lowry method.

B : Biuret method.





modification of the DEAE cellulose filter method of Schmidt and Raftery (1973). AChR (100 µl) in 10 mM potassium phosphate buffer, pH 7.4, containing 1% (v/v) Triton X100, and 0.1% (w/v) bovine serum albumin, was incubated with ^{125}I -α-BGT (1 - 1.5 nM; 50 µl) in the same buffer for 90 min at room temperature. Termination of the reaction was achieved by the addition of cold (4°C) buffer (1 ml). The sample was filtered through two DE 81 cellulose filter discs (2 cm diameter), and the discs were washed with buffer (5 ml). The washed discs were counted in an LKB 1280 Ultrogamma counter. Blank samples containing no AChR were used to determine non-specific binding, and AChR activity was expressed as the molarity of α-BGT binding sites.

^{125}I -α-BGT binding to chick myotube cultures

Incubation medium was removed from culture dishes, and each dish was washed three times with PBS (2 ml). To each dish was then added assay medium (1 ml; MEM, 20 mM HEPES, buffered to pH 7.2 with NaOH, containing 2 mg/ml bovine serum albumin), with or without decamethonium bromide (10^{-5} M final concentration), and the cultures were incubated at room temperature for 30 min. Following this, ^{125}I -α-BGT (10 - 60 µl to give a final concentration of 2.5 - 15 nM) was added to each dish, and the cultures were incubated for a further 60 min at room temperature. The assay medium was then removed, and each dish was washed three times with PBS (2 ml). The myotube monolayer in each dish was suspended with 0.1 M NaOH (2 x 0.75 ml) and the suspension counted in an LKB Ultrogamma counter. Non-specific binding was taken to be binding in the presence of 10^{-5} M decamethonium bromide. Specific binding was determined by subtraction of non-specific binding from the total bound radioactivity. All assays were performed on triplicate culture dishes.

^{125}I -α-BGT binding to human foetal myotube cultures

Culture medium was removed from culture dishes, and each dish was washed twice with 199 medium, buffered with 20 mM HEPES and containing 10% donor horse serum (assay medium). Following this, cultures were incubated in assay medium (1 ml) with or without decamethonium bromide (final concentration 10^{-3} M) for 45 min at room temperature. ^{125}I -α-BGT was then added to a final concentration of 10 - 30 nM, and the cultures were incubated for a further 60 min at room temperature. At the end of this incubation, each dish was washed three times with assay medium (2 ml). The cell monolayer was then solubilised with 0.1 M NaOH (1.5 ml). The radioactivity in the solubilised cell suspension was counted in an LKB 1280 Ultrogamma counter. Non-specific binding was taken to be the binding in the presence of 10^{-3} M decamethonium bromide.

Inhibition of ^{125}I -α-BGT binding to Torpedo AChR by F(ab')$_2$ and Fab fragments

Purified Torpedo AChR (100 µl) was incubated for 30 min at 22°C with immune or non-immune F(ab')$_2$ or Fab fragments (50 µl), prior to assaying for ^{125}I -α-BGT binding as described above. F(ab')$_2$ and Fab fragments were tested over the range of 0.01 - 1 mg/ml.

Inhibition of ^{125}I -α-BGT binding to chick myotube cultures by F(ab')$_2$ and Fab fragments

Myotube cultures were washed three times with PBS (2 ml), and then incubated overnight at 4°C in assay medium (see above; 1 ml) containing immune or non-immune F(ab')$_2$ or Fab fragments (10 - 100 µl). At the end of this incubation, each culture dish was washed three times with PBS (2 ml), before assaying the cultures for ^{125}I -α-BGT

binding activity as detailed above. ^{125}I -α-BGT was used at a final concentration of 10 - 15 nM in these experiments.

Determination of AChR turnover in chick myotube cultures

AChR turnover from chick embryo myotube cultures was determined in the absence or presence of immune $F(ab')_2$ fragments, at 4°C and 37°C. Cultures were labelled with ^{125}I -α-BGT in the presence or absence of decamethonium bromide (10^{-5} M), and each culture dish was then washed 7 times with PBS. Immune $F(ab')_2$ (300 μg in 1 ml of assay medium) or assay medium alone was then added to each dish, and the cultures were incubated at 4°C or 37°C. At various time intervals the medium (1 ml) plus a single wash with PBS (1 ml) was removed from duplicate dishes containing myotubes labelled with α-BGT in the presence of decamethonium, and duplicate dishes labelled with α-BGT alone, and counted in an LKB Ultrogamma counter.

^{125}I - $F(ab')_2$ and Fab binding to chick myotube cultures

Culture medium was removed from triplicate culture dishes, and each dish was washed three times with PBS (2 ml). To each dish was added assay medium (1 ml) containing ^{125}I - $F(ab')_2$ (0.25 - 2.5 μM final concentration) or ^{125}I - Fab (0.5 - 6 μM final concentration), and the dishes were incubated for 30 min at 37°C, then overnight at 4°C. At the end of the incubation the assay medium was removed, and the cultures were washed three times with PBS (2 ml). The myotube monolayer in each dish was suspended with 0.1 M NaOH (2 x 0.75 ml) and counted in an LKB Ultrogamma counter. Specific binding of ^{125}I - $F(ab')_2$ or Fab to AChR was taken to be the difference between binding of immune and non-immune fragments.

F(ab')$_2$ binding to Torpedo AChR

^{125}I - F(ab')$_2$ binding to purified Torpedo AChR was assayed by a DEAE - cellulose filter assay. AChR (0.1 - 2 pmol in 100 µl of 10 mM potassium phosphate buffer, pH 7.4, containing 1% (v/v) Triton X100 and 0.1% (w/v) bovine serum albumin (binding assay buffer)) was incubated with ^{125}I - F(ab')$_2$ (0.1 - 4 µM final concentration) for 2 h at 22°C, or overnight at 4°C. Termination of the reaction was by the addition of cold (4°C) assay buffer (1 ml). The sample was filtered through two DE 81 cellulose filter discs (2 cm diameter), and the discs were washed with assay buffer (5 ml). Blank samples contained no AChR. The washed discs were counted in an LKB Ultrogamma counter. Bound radioactivity in the blank samples was considered to represent non-specific binding.

Unlabelled F(ab')$_2$ fragments and affinity purified F(ab')$_2$ fragments were assayed by competition with parent ^{125}I - F(ab')$_2$ fragments for binding to Torpedo AChR. Receptor (50 µl; 0.1 pmol diluted in binding assay buffer) was preincubated for 90 min at 22°C with unlabelled F(ab')$_2$ fragments (50 µl) before addition of ^{125}I - F(ab')$_2$ fragments (10 pmol) and assay for binding as described above.

Mathematical analysis of binding data

Binding data of ^{125}I - F(ab')$_2$ to Torpedo AChR and myotube cultures was subjected to Scatchard analysis for determination of the dissociation constant (K_D) using the equation

$$\frac{[RL]}{[L]} = \frac{-1}{K_D}[RL] + \frac{1}{K_D}[R_o] \qquad (1)$$

where $[RL]$ = bound ligand

$[L]$ = free ligand

$[R_O]$ = initial receptor concentration

K_D = dissociation constant

The ratio of $\frac{[\text{Bound}]}{[\text{Free}]}$ ligand was plotted against $[\text{Bound}]$ ligand, and the K_D calculated from the gradient of the line. When half the receptor binding sites are occupied by ligand (ie $[RL] = \frac{1}{2}[R_O]$) then equation (1) reduces simply to

$$K_D = [L] \qquad (2)$$

An approximation of K_D can, therefore, also be made from the ligand concentration giving half maximal saturation of binding.

In addition the inhibition of ^{125}I - α-BGT binding to Torpedo AChR and myotube cultures by $F(ab')_2$ and Fab fragments were analysed by use of the Hill equation.

$$\log_{10}\left(\frac{B}{B_{max} - B}\right) = n \log_{10}[L] - \log_{10}K$$

where $B = [RL]$

B_{max} = maximum bound ligand

$[L]$ = free ligand

n = the Hill coeffieient

K = dissociation constant

In the case of ^{125}I - $F(ab')_2$ binding, B_{max} was taken to be the amount of bound ligand at saturation. For inhibition of α-BGT binding the fractional saturation $\left(\frac{B}{B_{max} - B}\right)$ was calculated from the inhibition curve, using the maximum inhibition as a measure of B_{max}.

A plot of $\log_{10}\left(\frac{B}{B_{max} - B}\right)$ against $\log_{10}[L]$ yields a

straight line with gradient, n, and x - intercept (B = half B_{max}) of $-\log_{10} \frac{K}{n}$

Inhibition of ^{125}I - $F(ab')_2$ binding to Torpedo AChR by α-BGT

Inhibition of ^{125}I - $F(ab')_2$ binding to Torpedo AChR by unlabelled α-BGT was performed by preincubation of AChR (0.1 - 2 pmol in binding assay buffer (100 µl)) with α-BGT (100 pmol) in the same buffer (10 µl), or with buffer alone, for 90 min at 22°C prior to assaying for ^{125}I - $F(ab')_2$ binding as described above.

Inhibition of ^{125}I - $F(ab')_2$ binding to chick myotube cultures by α-BGT

Myotube cultures were washed three times with PBS (2 ml). To each dish was then added assay medium (1 ml) containing α-BGT (10 - 20 pmol in 10 µl PBS) or PBS alone, and the cultures were incubated for 60 min at 22°C. Dishes were then washed twice with PBS (2 ml) prior to assaying for ^{125}I - $F(ab')_2$ binding as outlined above.

Inhibition of ^{125}I - $F(ab')_2$ binding to myotube cultures by purified Torpedo AChR

^{125}I - $F(ab')_2$ was preincubated with Torpedo AChR in 10 mM potassium phosphate buffer, pH 7.4 containing 0.1% (v/v) Triton X100, at molar ratios of 2.5:1 up to 50:1, or with buffer alone, at 4°C overnight, prior to assaying for ^{125}I - $F(ab')_2$ binding to myotube cultures. Binding of ^{125}I - $F(ab')_2$ was determined at a final concentration of 1 µM.

Physical and chemical modifications of Torpedo AChR

1) Heat inactivation: Torpedo AChR in 10 mM potassium phosphate

buffer, pH 7.4 containing 0.1% (v/v) Triton X100 was heated at 60°C for 10 - 30 min.

2) SDS - denaturation: AChR was boiled for 2 min in the presence of SDS (0.5 - 1% (w/v) final concentration).

3) Periodate oxidation: AChR (750 µl) was incubated for 4 h at 4°C in the dark with sodium metaperiodate (15 mM) in 50 mM sodium acetate buffer, pH 5.0, containing 1% (v/v) Triton X100 and 0.1% (w/v) bovine serum albumin (50 µl), or with buffer alone. At the end of the incubation the reaction was stopped by the addition of ethylene glycol (100 µl).

4) Glycosidase treatment: AChR was treated with a mixed glycosidase extract from Trichomonas foetus (T. foetus). The glycosidase extract has been found to contain fucosidase, mannosidase, galactosidase and hexosaminidase, but no protease activities (Watkins, 1966; Westwood et al., 1976). Lyophilised T. foetus extract was reconstituted at 4 mg/ml in 10 mM phosphate buffer, pH 6.4, containing 1% (w/v) bovine serum albumin. Torpedo AChR in the same buffer, containing 0.1% (v/v) Triton X100 but no bovine serum albumin, was incubated at 37°C for 4 h with reconstituted T.foetus extract (40 µl) or with reconstitution buffer alone (40 µl).

Binding of ^{125}I - $F(ab')_2$ to modified Torpedo AChR

Torpedo AChR was modified as described above. The modified receptor was assayed directly for ^{125}I - $F(ab')_2$ binding by the DEAE - cellulose filter method described above. In the SDS - denatured AChR preparation the final concentration of residual SDS was approximately 0.001%, and was considered to be negligible. Control tubes, therefore, contained no SDS. For T. foetus treated AChR the binding assay was

performed at 4°C to reduce any possible effect of glycosidase activity on the assay.

Inhibition of ^{125}I - $F(ab')_2$ binding to chick myotube cultures by modified Torpedo AChR

Modified Torpedo AChR was preincubated with ^{125}I - $F(ab')_2$ as described above for untreated receptor at a molar ratio of 1 to 5 (AChR to ^{125}I - $F(ab')_2$) prior to assaying for ^{125}I - $F(ab')_2$ binding to myotube cultures.

For heat-inactivated AChR, untreated receptor was used as the control. For SDS - denatured AChR the final concentration of residual SDS in the culture dish was approximately 0.05%, and hence this concentration of SDS was included in the control cultures with untreated AChR.

For periodate - treated AChR three different controls were set up.

1) AChR treated with periodate - oxidation buffer alone, with the addition of ethylene glycol.

2) ^{125}I - $F(ab')_2$ preincubated with periodate - oxidation buffer and ethylene glycol, as for periodate - oxidation of AChR prior to binding assay.

3) ^{125}I - $F(ab')_2$ preincubated with sodium metaperiodate and ethylene glycol, as for periodate - oxidation of AChR, prior to binding assay.

For glycosidase - treated AChR three different controls were again used.

1) AChR treated with buffer alone at 4°C or 37°C.

2) AChR treated with T. foetus extract at 4°C, under the same conditions as for preincubation with receptor at 37°C.

3) ^{125}I - $F(ab')_2$ preincubated with T. foetus at 37°C, as for preincubation of AChR, prior to binding assay.

Carnitine uptake by myotube cultures

Tritiated (^{3}H-methyl) carnitine (^{3}H-C; specific activity 1-2 Ci/mmol (Amersham International)) was diluted in 199 medium, containing 5% foetal calf serum (incubation medium) to a final concentration of 1 μM. For the ^{3}H-C uptake assay, chick myotubes were grown in 35 mm diameter collagen-coated petri dishes for 6-8 days as described in the methods section. Human myotube cultures were grown in 35 mm diameter, collagen-coated petri dishes in 199 medium, containing 10% donor horse serum for 14-21 days. The cultures were then washed once with incubation medium (2 ml), and ^{3}H-C (1 nmol) in incubation medium (1 ml) was added to each dish. The cultures were then incubated at 37°C under an atmosphere of 5% CO_2 in air for 18 h. At the end of this incubation each dish was washed twice with incubation medium, and finally fresh incubation medium (0.9 - 1 ml) was added to the cultures prior to assaying for myolysis (see below).

Serum-mediated muscle cell lysis

To measure the lysis of myotubes caused by normal human sera, or sera from myasthenic patients, myotube cultures were first labelled with ^{3}H-C as described above. Serum (2.5 - 10%, v/v) was then added to triplicate culture dishes to give a final incubation volume of 1 ml, and the cultures were incubated at 37°C for 5 h. Triplicate control cultures received no serum. Following this incubation, each culture was washed twice with incubation medium (2 ml), and the cell monolayer was solubilised with 0.1M NaOH (1ml). The solubilised cell suspension was transferred to scintillation vials, and NE 260 scintillation fluid (New England Nuclear; 10ml) was added to each vial. The vials were briefly mixed on a vortex mixer, and the radioactivity in the solubilised cell suspension was counted by using a Packard liquid scintillation counter.

scintillation counter.

Serum - mediated myotoxicity was calculated by using the formula

$$\text{Myotoxicity} = 100 - \frac{ER^{3}H - C}{TR^{3}H - C} \times 100$$

where $ER^{3}H - C$ is the retained $^{3}H - C$ in the presence of test serum, and $TR^{3}H - C$ is the retained $^{3}H - C$ in the absence of serum.

Absorption of sera with chicken liver homogenate

Chicken liver was washed free of blood with PBS, diced, and re-washed with PBS. The diced liver was mixed 1:1 with cold (4°C) PBS and homogenised using a Potter-Elvsham homogeniser. The homogenate was centrifuged at 10,000 g for 15 min. The pellet was retained and stored at -20°C for further use.

Absorption of sera was carried out by stirring each sample (1 ml) of serum with thawed homogenate (0.5 ml) for 2 h at room temperature. The serum was recovered by centrifuging at 10,000 g for 30 min.

Heat inactivation of sera, and effect of complement on myolysis

Sera were heat inactivated by incubation at 56°C for 30 min. To test the effect of complement on serum - mediated myotoxicity, the heat - inactivated serum (2·5% v/v), with or without guinea pig complement (Miles Laboratories) in commercial diluent (10% v/v), was used in the myotoxicity assay described above.

RESULTS

Preparation of chick embryo myotube cultures

Dissociation: Limb muscle from 10 - 11 day incubated chick embryos was routinely dissociated by incubation with 0.1% trypsin. Cell aggregates were rare with this procedure, and were removed by passage of the cell suspension through two 50 μm pore-size nylon discs. Yields of nucleated cells were regularly in the range 150 - 200 x 10^6 cells per gram wet weight of muscle.

Initial plating densities ranged from 1 x 10^5 - 1 x 10^7 cells/35 mm dish. At the lower densities the onset of fusion was significantly delayed, whereas, at high densities, overcrowding in the cell monolayer led to rapid overgrowth with fibroblasts prior to myoblast fusion, and extensive cell death, with rounding-up and detachment of cells from the substratum. Thus an initial seeding density of 1 x 10^6 cells/dish proved optimal for growth and differentiation of muscle cells.

Growth and Differentiation: Mononucleated cells which settled onto the collagen substratum were at first round in shape, with bipolar or multipolar morphologies appearing after about 24 h in culture (Figure 10a). By day 2 in culture, myoblast fusion was extensive (Figure 10b) with small, multinucleated, elongated cells present, some of which exhibited extensive branching. Myoblast fusion appeared to occur in approximate synchrony, and was essentially complete by day 3 in culture. By day 5 large myotubes had formed, and were abundant in the culture (Figure 10c). Nuclei in myotubes were arranged in rows, at first centrally placed, but later moving laterally to become sited under the plasma membrane (Figure 11).

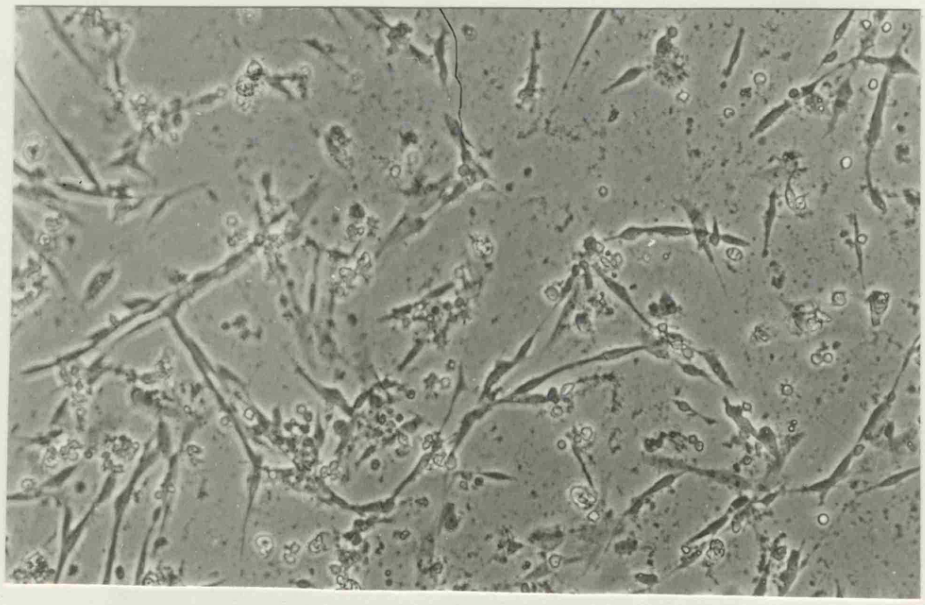
FIGURE 10.

(a) Phase contrast micrograph of mononucleated cells in a 24h culture of dissociated chick embryonic muscle seeded at $1x10^6$ cells/35mm dish.

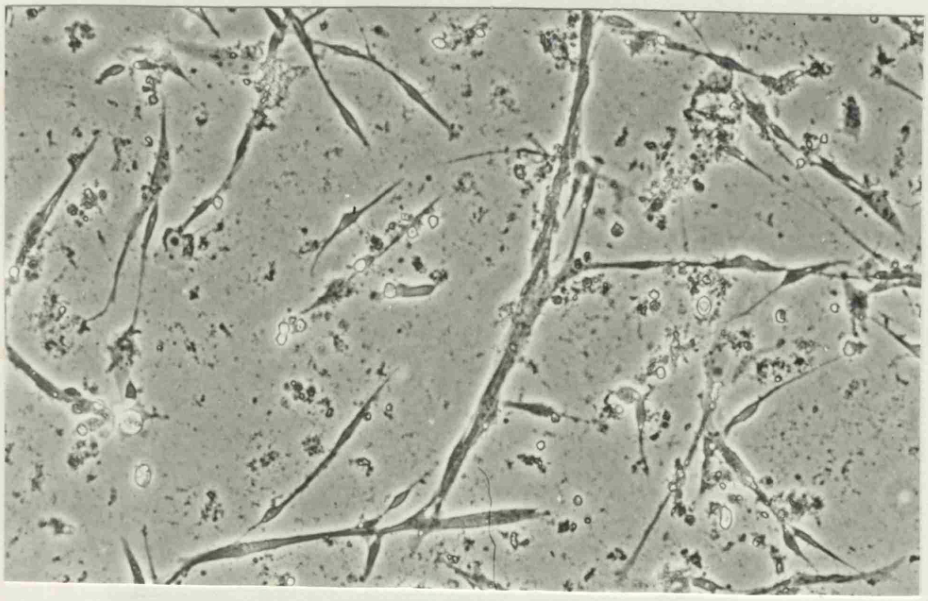
(b) Occurence of cell fusion in a 48h culture of chick embryonic muscle seeded at $1x10^6$ cells/35mm dish. Numerous cell-cell contacts can be seen, with myoblasts aligning lengthwise. Multiple cell fusions are also apparent.

(c) Phase contrast micrograph of a 5-day culture of chick embryonic muscle showing well formed multinucleate myotubes.

a



b



c

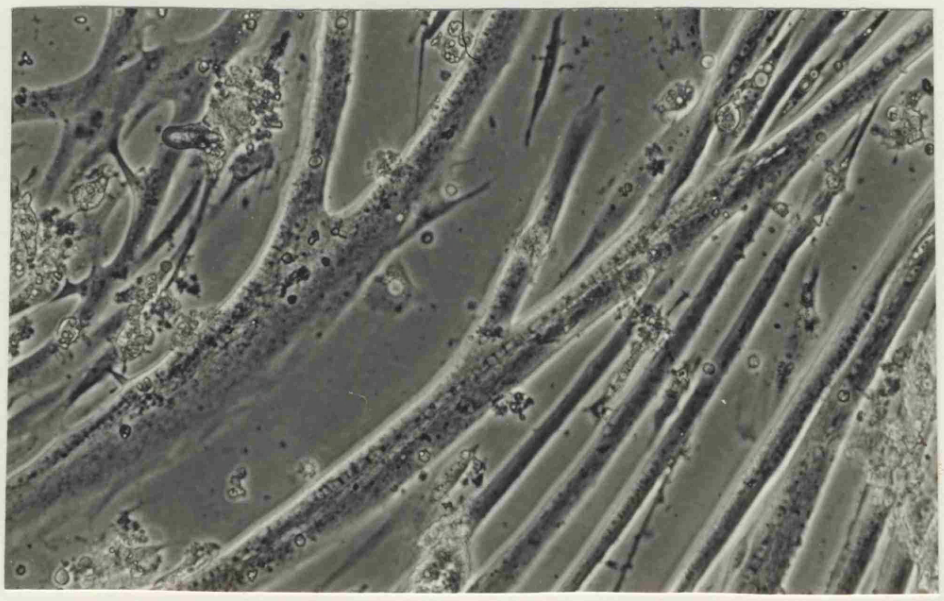
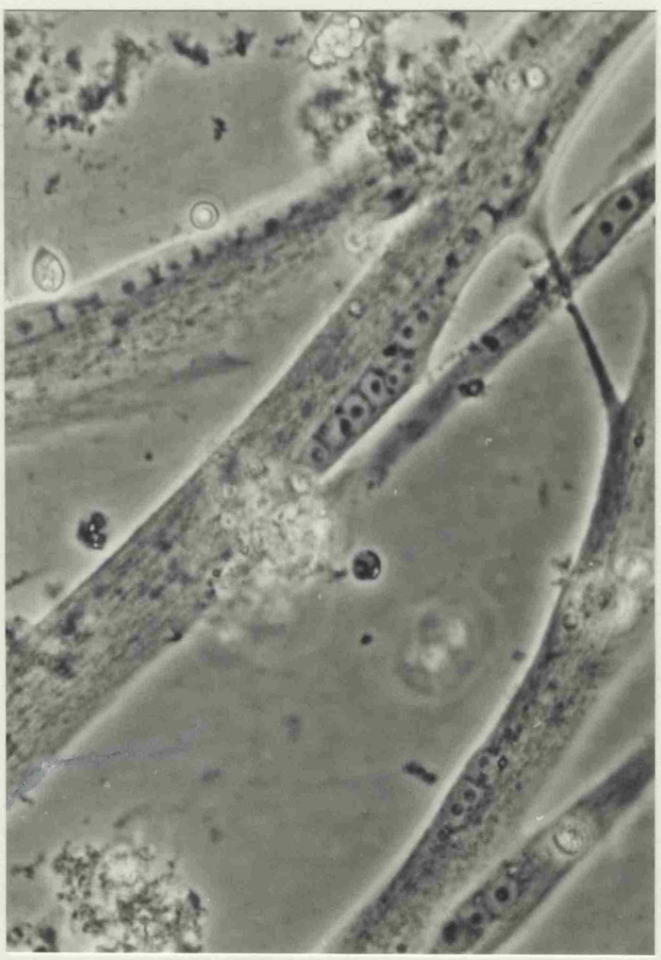


FIGURE 11. A 7-day culture of chick embryonic muscle showing the arrangement of nuclei along the periphery of the myotubes.



Cross-striations were also found from day 5 onwards (Figure 12). Spontaneous contractions, rhythmical or single, could be observed in cultures containing more mature fibres.

Rapidly dividing mononuclear cells in the cultures were killed by exposure to a DNA-synthesis inhibitor, cytosine arabinoside (ara C). The fused myoblasts were post-mitotic, and hence unaffected by the drug. Cultures treated with ara C were essentially free of contaminating mononuclear cells (Figure 13b). By contrast, cultures not exposed to the drug became overgrown with mononuclear cells by day 5 in culture (Figure 13a). It is not possible to say whether the contaminating mononuclear cells were fibroblasts or unfused myoblasts on the basis of their morphology alone.

Protein estimation of chick myotube cultures

The total protein content of chick embryo myotube cultures was determined by the method of Lowry et al. (1951). Initial experiments were performed to determine the minimum extraction time with 0.1 M NaOH needed to solubilise completely the myotube monolayer. The results are shown in Table 6, and demonstrate that a 30 min extraction was sufficient for efficient solubilisation.

Table 7 shows the cell protein content of 20 culture dishes from a single myotube culture, extracted with 0.1 M NaOH for 30 min. The mean cell protein was 255 µg/dish with a standard deviation of 20 µg/ml, representing 7.8% of the mean. The mean protein content of cell-free dishes was 30 µg/ml, probably representing serum components non-specifically adsorbed onto the collagen matrix. Routine protein estimations on chick myotube cultures during the course of this study were performed on triplicate cultures, and used a 30 min extraction time with 0.1 M NaOH. The mean protein content from culture to culture was variable, ranging from 188 - 576 µg/dish, with an overall mean $\pm$ standard deviation (S.D.) of 355 $\pm$ 118 µg/ dish (n = 17).

FIGURE 12. Mature myotubes in a 9-day culture of chick embryonic muscle.

Cross-striations can be observed in the large myotube at the right of the field.

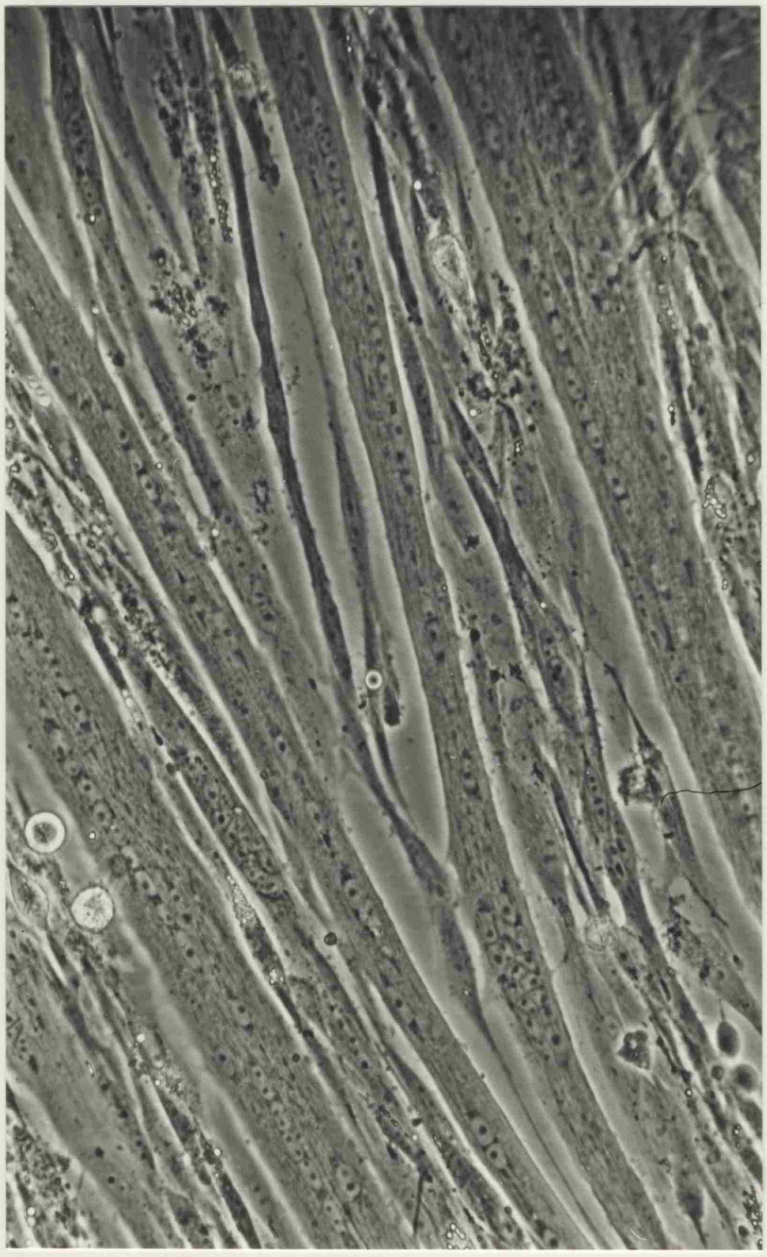
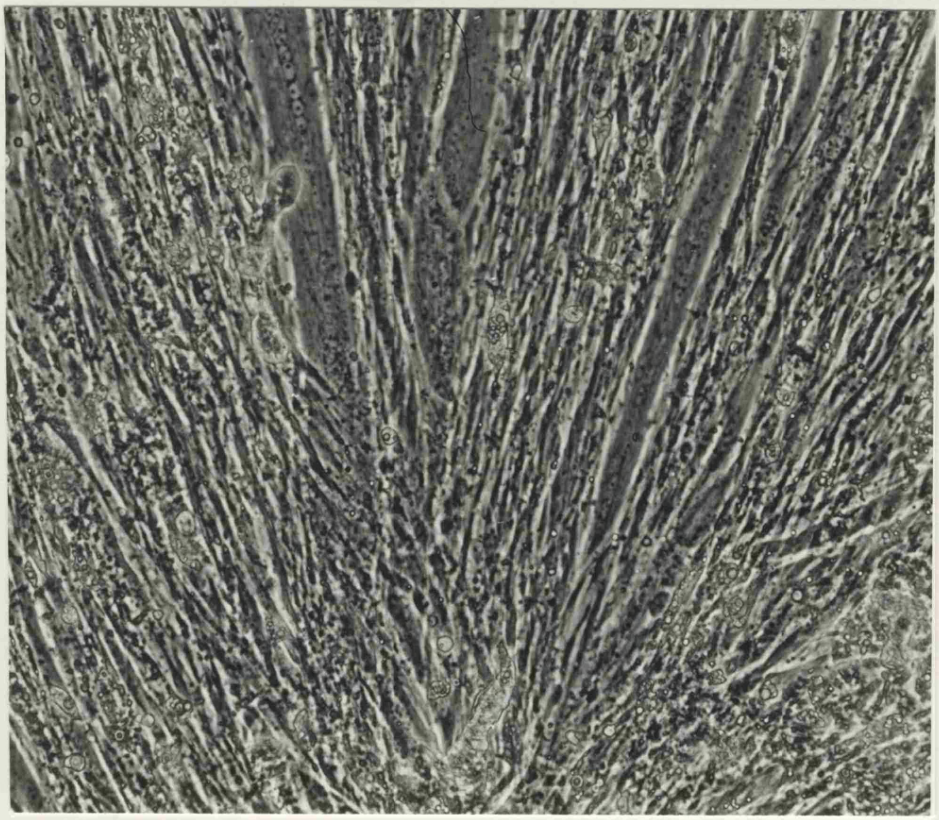


FIGURE 13. The effect of cytosine arabinoside (ara c) on a culture of chick embryonic muscle.

(a) Untreated. Mononuclear cells have occupied all the spaces between the myotubes, and have also partially overgrown the myotubes themselves.

(b) Treated with ara c. Ara c was added at 52h in culture, and was incubated with the cells for 60h as detailed in the Methods (p 71). A relatively pure population of myotubes resulted from this treatment.

a



b

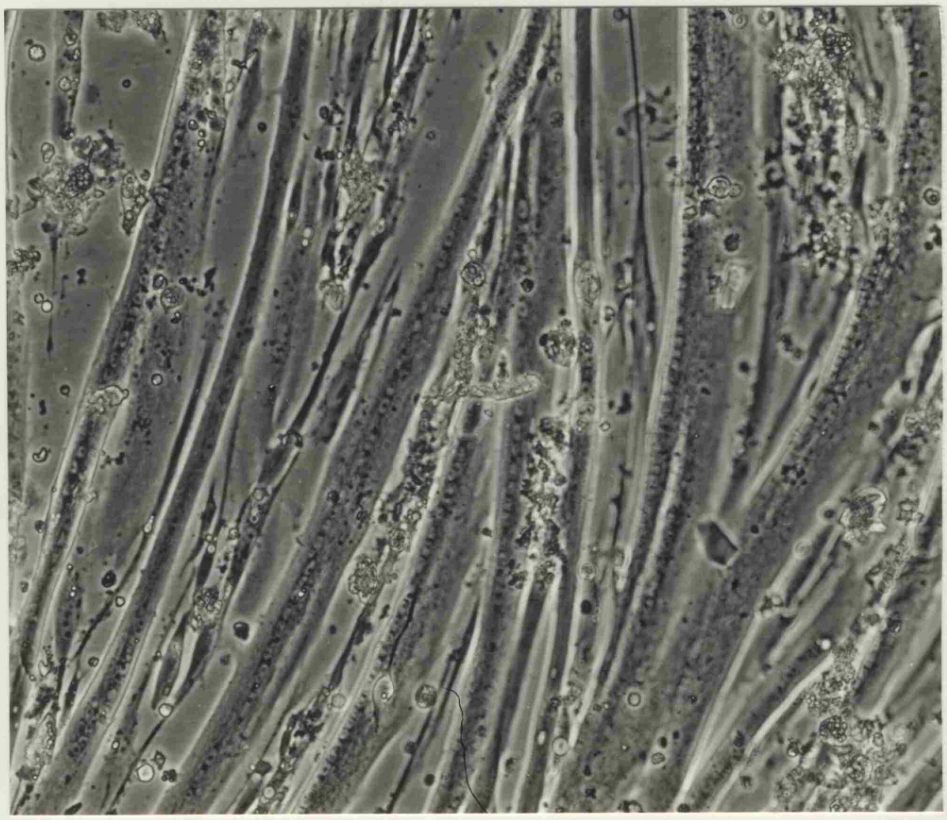


TABLE 6. The effect of extraction time on determination of the cell protein content of a culture of chick embryonic muscle.

Culture dish	Cell protein content (µg/dish)
1	165
2	170
3	165
4	155
5	165
6	180
7	165
8	155
9	140
10	195
11	175
12	155
13	155
14	225

Dishes 1-7 were extracted with 0.1M NaOH for 30min.

Dishes 8-14 were extracted with 0.1M NaOH for 60min.

TABLE 7. Variation in cell protein content of 20 culture dishes within a single culture of chick embryonic muscle.

Culture dish	Cell protein content (µg/dish)
1	240
2	285
3	280
4	255
5	285
6	250
7	290
8	250
9	260
10	260
11	250
12	235
13	235
14	250
15	240
16	285
17	250
18	240
19	250
20	220

Preparation of human foetal myotube cultures

Limbs were obtained from foetuses of 8 weeks gestation or older. Below this age identification of limbs was impossible. Preservation of intact limbs was greater with increasing foetal age.

Disaggregation: The disaggregation of human foetal muscle was much more difficult than that of chick embryo muscle. After 30 min exposure to 0.1% (w/v) trypsin large tissue lumps were still present, with a low yield of single nucleated cells. Much more complete dissociation was achieved by a 60 min exposure to the enzyme (Table 8). When the single cell suspension obtained was plated out into collagen-coated 35 mm tissue culture dishes (5×10^5 cells/dish) the plating efficiency (the number of cells present after 24 h *in vitro* culture, expressed as a percentage of the initial plating density) was similar whether a 30 or 60 min exposure to trypsin was employed (Table 8).

Disaggregation could be achieved by using either 0.1% (w/v) trypsin or 0.5% (w/v) collagenase. Cell yields were slightly higher with a 1 h exposure to trypsin (Table 9). Plating efficiency was also equal, or higher with trypsin (Table 9). For this reason, 0.1% trypsin was used routinely in the preparation of single cell suspensions from foetal muscle. Mechanical disaggregation of muscle resulted in a much lower cell yield, and plating efficiency (Table 9).

Preplating of muscle cell suspensions: Cell suspensions prepared from human foetal muscle contained both myoblasts and fibroblasts (see Figure 14a). An attempt was made to enrich the cell suspension in myoblasts by preferential attachment of the fibroblasts to tissue culture flasks, following the method of Yaffe (1968). In this study between 10 and 30% of cells attached to the flask during the preplating procedure. Examination of the attached cells after 24 h incubation

TABLE 8. Time-dependence of tryptic digestion of human foetal muscle.

INCUBATION TIME	CELL YIELD ($x10^5$/mg tissue)	VIABILITY (%) *	PLATING EFFICIENCY (%) †
30min.	0.3	97	9.6
60min.	1.4	100	10.0

* Viability was determined by Trypan Blue exclusion.

† To determine the plating efficiency, 5×10^5 viable nucleated cells were plated out into 35 mm diameter collagen-coated petri dishes in 199 medium containing 10% donor horse serum. The plating efficiency was calculated as the number of cells attached to the collagen substratum at 24h *in vitro*/number of cells initially plated.

TABLE 9. Disaggregation of human foetal muscle tissue.

Preparation/ Foetal age	Disaggregation method	Cell yield ($x10^5$/mg tissue)	Viability (%)	Plating efficiency (%)
1 11 weeks	Mechanical	0.35	88.5	-
	Collagenase/ DNAse	2.5	100	-
	Trypsin	3.1	100	-
2 10 weeks	Mechanical	0.35	82	0.25
	Collagenase/ DNAse	2.8	100	5.2
	Trypsin	3.1	100	5.2
3 14 weeks	Mechanical	0.64	81.6	0.3
	Collagenase/ DNAse	1.9	100	5.2
	Trypsin	1.9	100	10.6

Viability and plating efficiency were calculated as in Table 8.

at 37°C showed that all the cells had a fibroblastic morphology. The plating efficiency of unattached cells was essentially identical to that of the original cell suspension.

Growth and differentiation: After plating, cells settled on to the collagen substratum. Most cells appeared spherical when viewed by phase-contrast microscopy. By 24 h *in vitro* some of the attached mononuclear cells had processes on either side, so that an elongation of the cell took place (bipolar morphology). Mononucleated cells with more than two processes were also present in the first days in culture (multipolar morphology). The two types of cell are shown in Figure 14. It was not possible, however, to distinguish unfused myoblasts from fibroblasts on the basis of their morphology. By 48 h in culture mononucleated cells had increased in size. Limited contact between cells was visible, with cells of both types being involved (Figure 15). By day 3 *in vitro* cell contacts were extensive, with multiple fusions being common (Figure 16a,b). Long processes extending from one cell to another were also observed, with the myoblasts aligning end to end. By day 7 multinucleated cells could be seen, and myotubes were well developed by day 14 (Figure 17). The myotubes contained three or more nuclei arrayed in rows either centrally or laterally under the cell membrane (Figure 18a,b), or in closely-packed groups (Figure 18c). Some myotubes were extensively branched (Figure 19).

Reduction of fibroblast growth in cultures: When cultures were grown in medium containing 10% foetal calf serum, mononuclear cells often overgrew the cultures by 10 days (Figure 20a). Preplating alone had no effect on this overgrowth (Figure 20b). Three different cytotoxic drugs were used in an attempt to reduce the overgrowth

FIGURE 14.

H&E stained preparation showing mononucleated cells of varying morphologies in a 24h culture of enzymatically dissociated human foetal muscle. Seeded at $5x10^5$/35mm dish, in 199 medium, containing 10% donor horse serum.

B: bipolar morphology

M: multipolar morphology

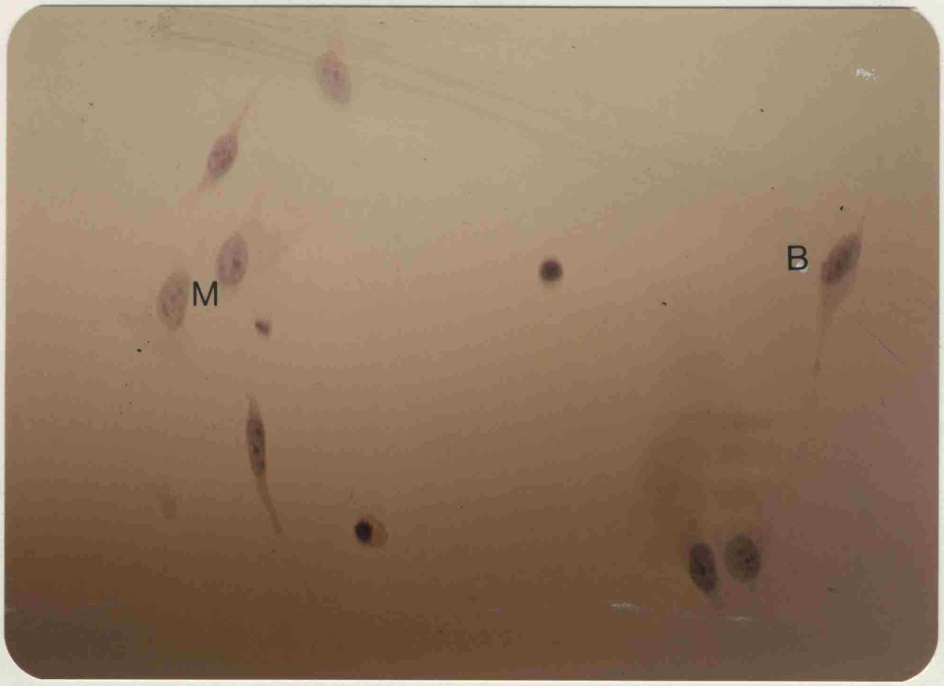

M
B

FIGURE 15. Onset of fusion in a 2-day culture of human foetal muscle. Cells of both bipolar and multipolar morphologies are seen to exhibit apparent contact. Seeded at $5x10^5$ cells/ml.

FIGURE 16.

Apparent contact between mononucleated cells in a 3-day culture of human foetal muscle. In (a) long processes extending from one cell toward another can be seen, with some of the processes actually in contact with each other. Multiple cell fusions can be seen, such as that running from top right to bottom left of the field involving at least 6 cells. At higher power -(b)- it can be seen that cells of all morphologies were involved in this fusion.

a

b

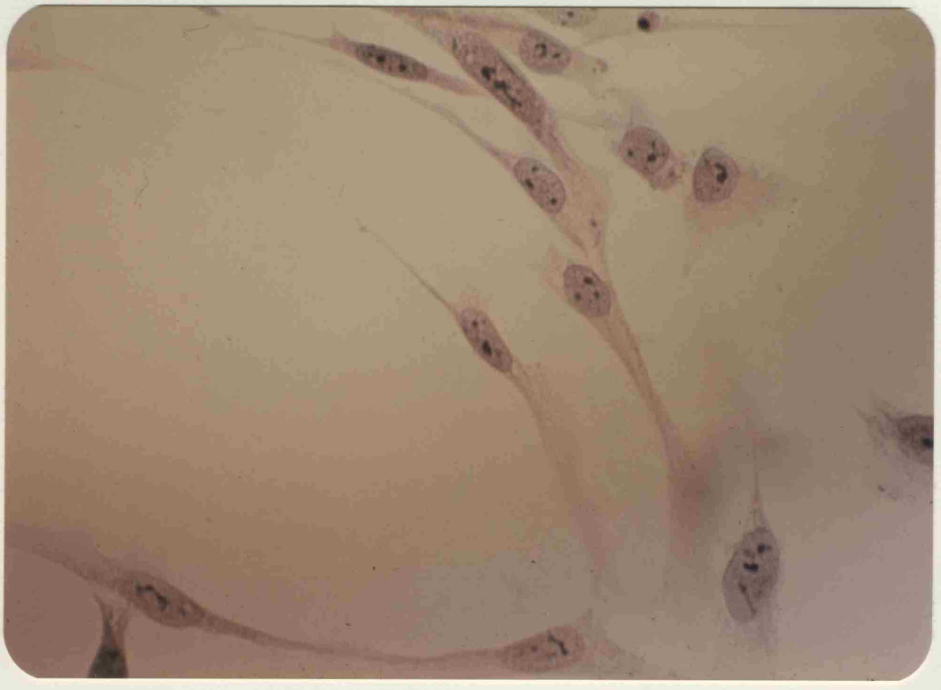


FIGURE 17. Appearance of myotubes at day 14 in a culture of human foetal muscle. Seeded at $5x10^5$/ml.

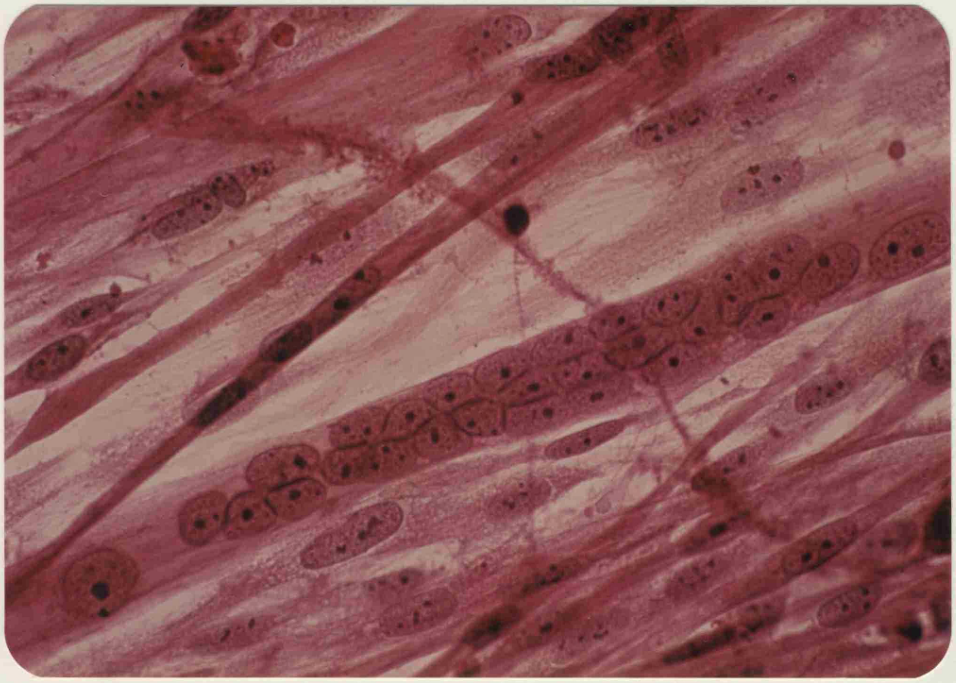


FIGURE 18. Arrangement of nuclei in myotubes of a 14-day culture of human foetal muscle.

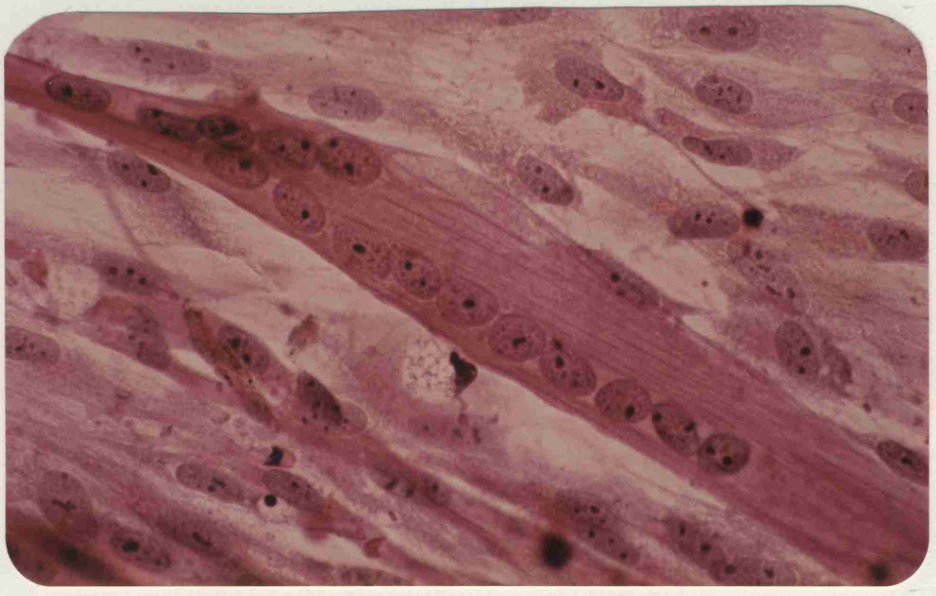
(a) Central

(b) Peripheral

(c) Clustered

a

b



c

FIGURE 19. An extensively branched myotube in a 14-day culture of human foetal muscle.

of cultures (Table 10). Vincristine caused extensive cell death, with most cells "rounding up" and detaching from the substratum. Both adriamycin and cytosine arabinoside were successful in reducing the fibroblast density of cultures when added at day 3 (Figure 20c,d). However, due to the relatively long time-scale of myotube formation, many myoblasts were also killed, resulting in little enrichment of the cultures. When the drugs were added later than day 3 (to allow more extensive myoblast fusion) they were ineffective in checking fibroblast growth. Because of this cytotoxic drugs were not routinely employed in the preparation of human myotube cultures.

Cultures grown in medium containing 10% donor horse serum had much less fibroblastic contamination, and myotube formation was extensive (Figure 20e,f).

Effect of media formulation on growth of myotube cultures: The various media, and supplements used in the cultivation of human foetal myotube cultures are detailed in Table 11. Changing the medium had little effect on culture growth, and medium 199 was chosen for routine preparation of myotube cultures and studies of myolysis. The presence of 2.5% chick embryo extract was found to be toxic to human myotube cultures. As has already been stated, the addition of horse serum resulted in least fibroblastic contamination of the cultures.

Effect of initial plating density on growth of myotube cultures

The initial plating density ranged from 1×10^4 - 2×10^6 cells/culture dish. At 1×10^5 cells/dish or less no growth or fusion occurred. Myotube formation occurred at all concentrations greater than this, with optimal growth and differentiation occurring when cells were seeded at 1×10^6 cells/dish in 2 ml of medium.

TABLE 10. Cytotoxic drugs used in the attempt to reduce the contamination of human foetal muscle cell cultures by fibroblastic cells.

DRUG	CLASS	CONCENTRATION & TIME TESTED	PRIMARY MODE OF ACTION	SCHEDULE DEPENDENCE OF CELL KILLING	REFERENCE
Cytosine arabinoside	Anti-metabolite	$1x10^{-5}/1x10^{-4}$M 48 - 60h	Inhibition of DNA polymerase by cytosine arabinoside triphosphate (ara-CTP).	Maximum sensitivity in S-phase of cell cycle.	Chabner, 1981.
Adriamycin	Anthracycline	500ng/ml 48 - 60h	Inhibition of normal nucleic acid synthesis and metabolism by interaction with cellular DNA.	All phases of cell cycle.	Du Vernay, 1981.
Vincristine	Vinca-alkaloid	1µg/ml 48 - 60h	Inhibition of tubulin polymerisation into microtubules resulting in arrest of cells in metaphase.	All phases of cell cycle, but effect may not be apparent until cell enters mitosis.	Johnson, 1982.

FIGURE 20.

(a) 10-day culture of human foetal muscle grown in medium containing foetal calf serum. The culture is completely overgrown by mononuclear cells.

(b) A similar culture to (a) after preplating of the dissociated cell suspension according to the procedure of Yaffe (1968). No reduction in the mononuclear cell contamination was achieved.

(c) 10-day culture of human foetal muscle after previous exposure to adriamycin. The drug has killed both myoblastic and fibroblastic cells, resulting in a low density mixed culture. Subsequent regrowth resulted in overgrowth as in (a).

(d) 10-day culture of human foetal muscle after previous exposure to cytosine arabinoside. Some selective kill of mononuclear cells has occurred, resulting in a culture enriched in myotubes. Remaining mononuclear cells however , regrew and myotubes were arrested at an early stage of maturation.

(e) 10-day culture of human foetal muscle grown in medium containing horse serum. Selective inhibition of mononuclear cell growth has occurred, resulting in a relatively pure population of myotubes.

(f) The same culture as (e) shown at 14 days. The small multinucleated cells have developed into well-differentiated myotubes.

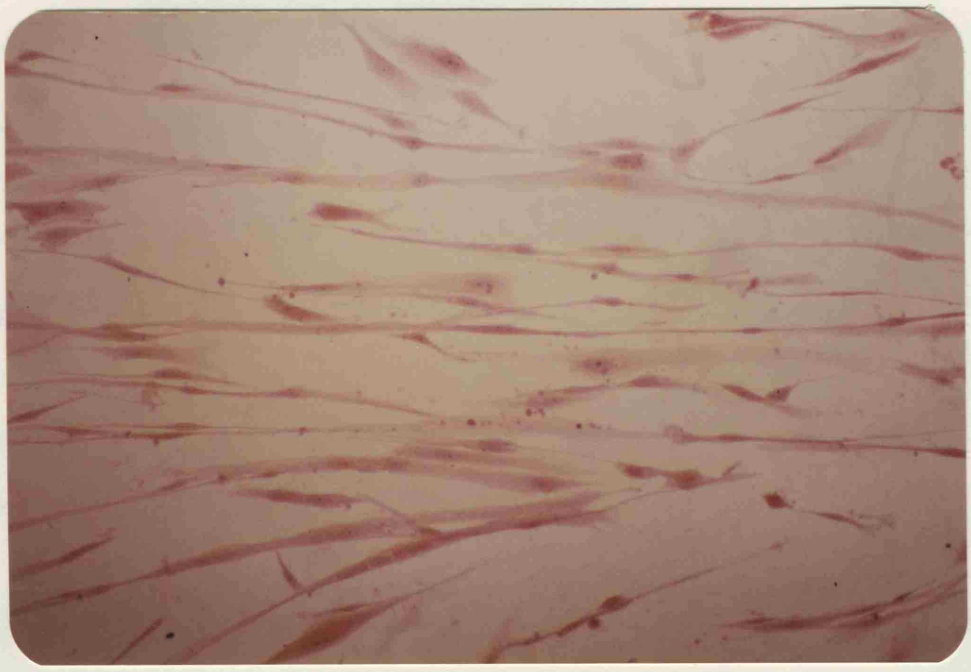
a

b

c

d

e



f

TABLE 11. Media and medium supplements used in the preparation of human foetal myotube cultures.

Medium	Supplement
RPMI 1640	10% foetal calf serum ; 10% horse serum
Eagles minimal essential medium	10% foetal calf serum ± 2% chick embryo extract
McCoys 5A	10% foetal calf serum
Medium 199	10% horse serum

Preparation of AChR from Torpedo marmorata

Table 12 shows a typical purification scheme for AChR from frozen Torpedo electric organ. The specific activity of the purified AChR ranged from 2 - 10 nmoles toxin binding sites/mg protein (7 preparations). The purity and subunit composition of the final AChR preparation was assessed by SDS polyacrylamide gel electrophoresis under reducing conditions. The gel pattern obtained (Figure 21) was similar to those in previous reports of purified Torpedo AChR (Barnard et al., 1979; Lindstrom et al., 1979; Raftery et al., 1979), with four major bands at 40000, 50000, 57000 and 64000 daltons.

Preparation and characterisation of ^{125}I -α-BGT

Figure 22 shows a typical elution profile of ^{125}I -α-BGT from Sephadex G 25. The ratio of α-BGT to ^{125}I in the reaction mixture was approximately 3 to 1, and incorporation of the total ^{125}I added to the reaction vessel into α-BGT averaged 91 $\pm$ 3% (mean $\pm$ S.D., n = 10 determinations). The specific activity of the labelled toxin was 733 $\pm$ 8 Ci/ mmol (n = 10). The biological activity of the labelled α-BGT was calculated by using the formula

$$\text{Biological activity} = \frac{\text{Total } ^{125}I \text{ bound} - ^{125}I \text{ bound in blank}}{\text{Total } ^{125}I \text{ added}} \times 100\%$$

A filter blank which contained buffer and ^{125}I -α-BGT but without AChR retained 4.6 $\pm$ 0.9% (n = 4) of the total radioactivity added to the assay sample. The biological activity of the ^{125}I -α-BGT was 56 $\pm$ 5% (n = 5) with a range of 41 - 75%. It was found that 72 $\pm$ 1% (n = 5) of the total radioactivity in the labelled toxin was precipitated by 6% (w/v) trichloroacetic acid.

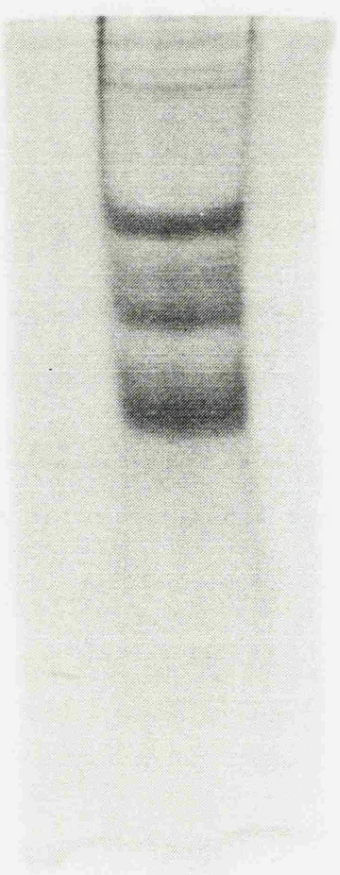
The iodinated α-BGT was separated into mono - and di-iodinated

TABLE 12. Typical purification scheme for AChR from Torpedo marmorata electric organ.

Stage of Preparation*	Total protein (mg)	AChR activity (nmol of α-BGT bound	Specific activity (nmol/mg)	Recovery of α-toxin binding activity relative to the homogenate (%)
Homogenate	2500	500	0.2	100
Supernatant II (Triton X100) extract)	430	215	0.5	43
Bound fraction after elution from toxin-Sepharose affinity beads.	38	190	5.0	38
Final AChR preparation	21	140	6.5	28

*Stages of preparation are as shown in Figure 8 (p.80; Methods section).

FIGURE 21. An SDS polyacrylamide electrophoresis gel of purified AChR from Torpedo marmorata.



species by the method of Vogel et al. (1972). Figure 23a shows the elution profile from CM-Sephadex C 50 of a typical, routine preparation of ^{125}I -α-BGT. The major peak (Peak IV), containing 69% of the bound radioactivity, was considered from its elution position to be mono-iodo α-BGT (Vogel et al., 1972). Peak III was similarly taken to contain di-iodo toxin. The molar ratio of mono - to di-iodo α-BGT in the preparation was calculated to be approximately 6.4:1. Peak II may represent aggregated ^{125}I -α-BGT (Jailkhani et al., 1984), while peak I consisted of either ^{125}I non-covalently associated with α-BGT or completely denatured ^{125}I -α-BGT (see below). The recovery of radioactivity from the ion-exchange column was close to 100%.

For preparation of mono - and di-iodo α-BGT the iodination procedure was performed with 100 μg of α-BGT. The elution profile of this ^{125}I -α-BGT preparation from CM-Sephadex C 50 is shown in Figure 23b. The ratio of mono - to di-iodo toxin was 1.6:1. The increased proportion of di-iodo α-BGT probably resulted from a higher concentration of chloramine - T in the scaled up iodination reaction (1.67 mg/ml compared with 1 mg/ml in the routine preparation).

Three fractions were collected from the column eluate (Figure 23b). Fractions 2 and 3 were considered to be di - and mono-iodo α-BGT respectively. The concentration of iodinated toxin in the two peaks was calculated by using the parameters shown in Table 13. By these means a concentration of 62 pmol/ml was calculated for di-iodo toxin, and 116 pmol/ml for mono-iodo toxin.

The three fractions were further characterised by binding to Torpedo AChR. A maximum of only 10% of fraction 1 was bound to AChR, suggesting that it consisted largely of non-covalently associated ^{125}I, or denatured and biologically inactive ^{125}I -α-BGT. Excess

FIGURE 22. Gel filtration of ^{125}I-α- bungarotoxin (BGT) on Sephadex G-25.

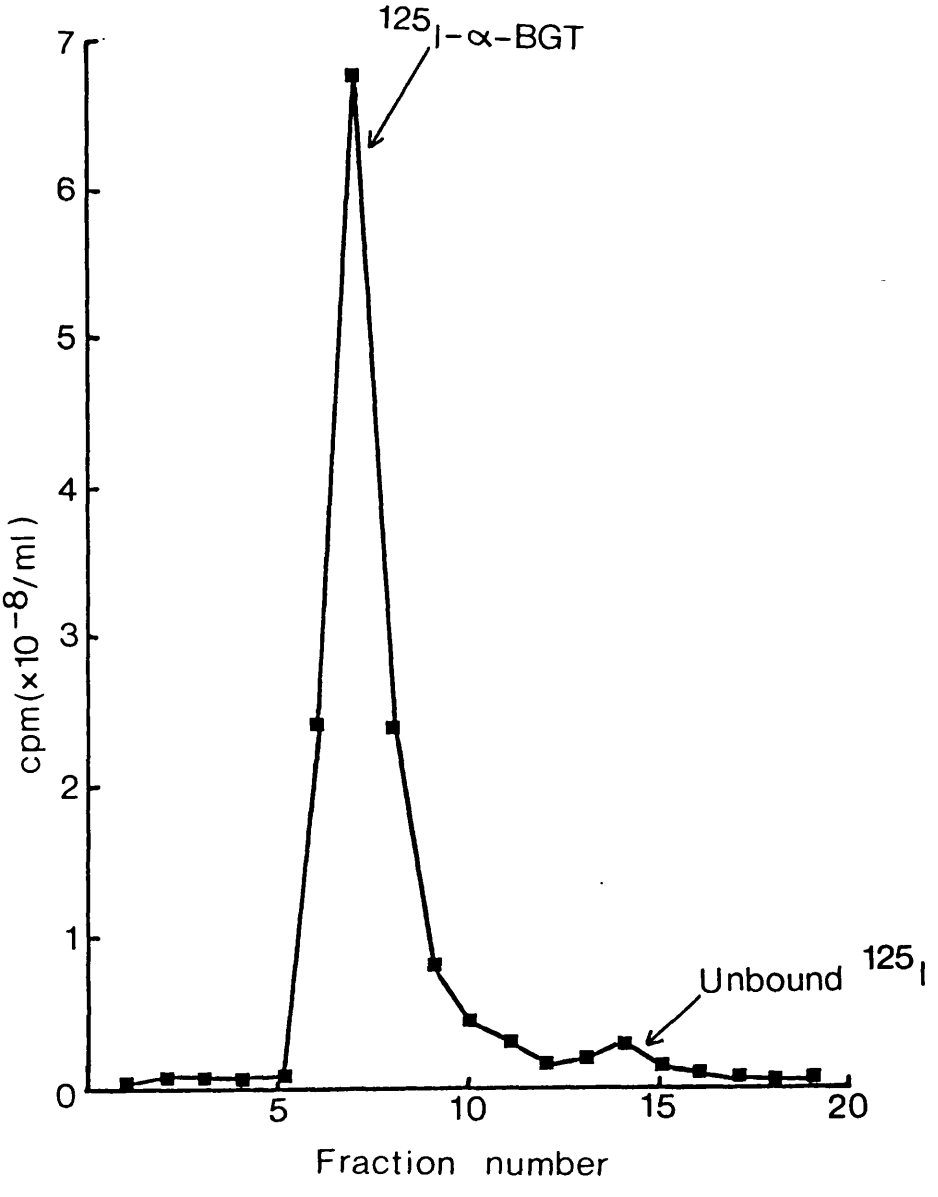


TABLE 13. Parameters used in calculating the concentration of mono- and di-iodo α- bungarotoxin eluted from CM-Sephadex C50.

Specific activity of $Na^{125}I$ (mCi/μatom I)	=	2320
D.p.m./μCi radioactivity	=	2.2×10^6
∴ D.p.m./μatom I	=	5.1×10^{12}
Efficiency of γ-counter	=	60%
Atoms I/molecule of α- bungarotoxin	=	2 (di-iodo toxin) 1 (mono-iodo toxin)

FIGURE 23a. Fractionation of ^{125}I-α- Bungarotoxin (10µg) on CM - Sephadex C50.

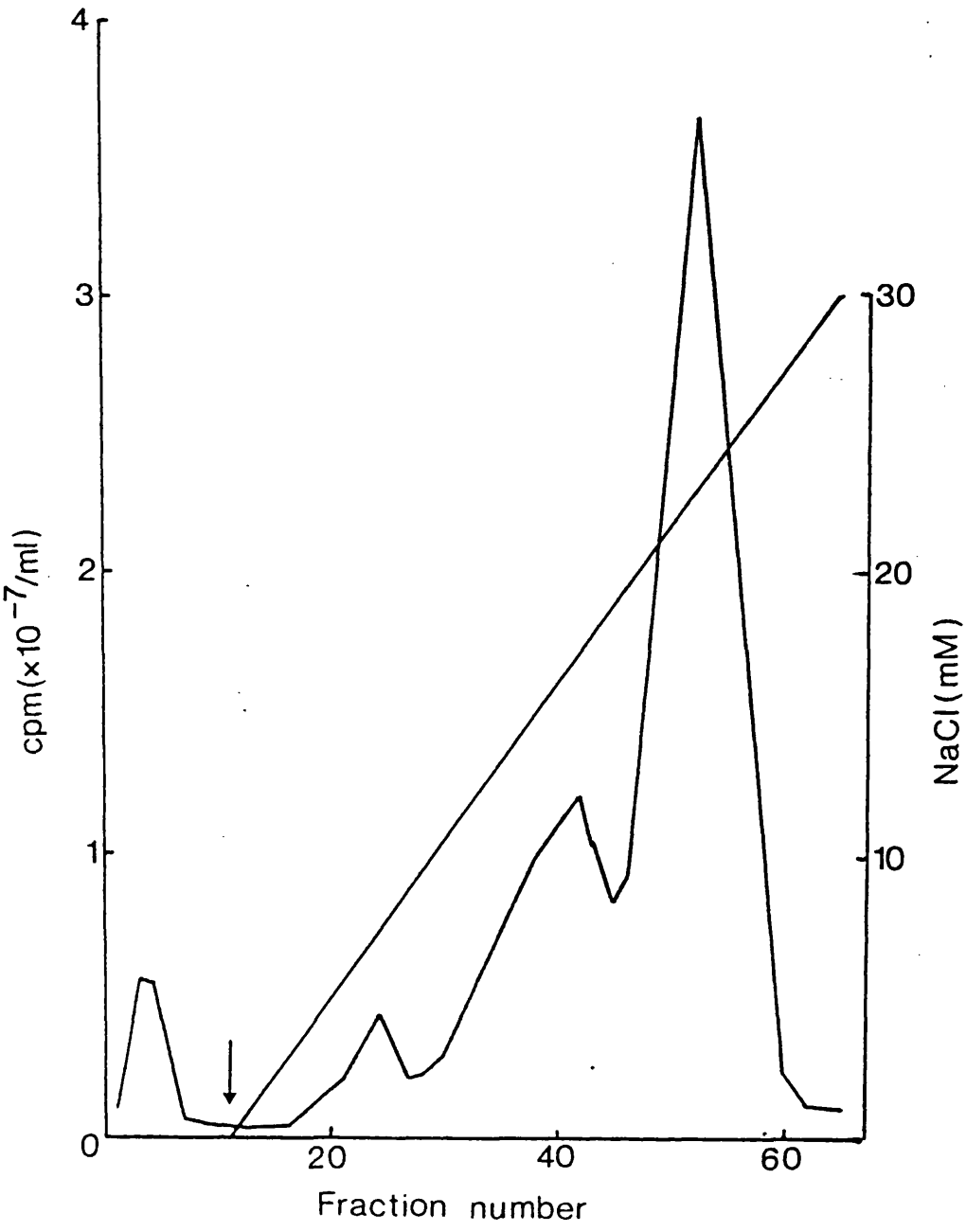
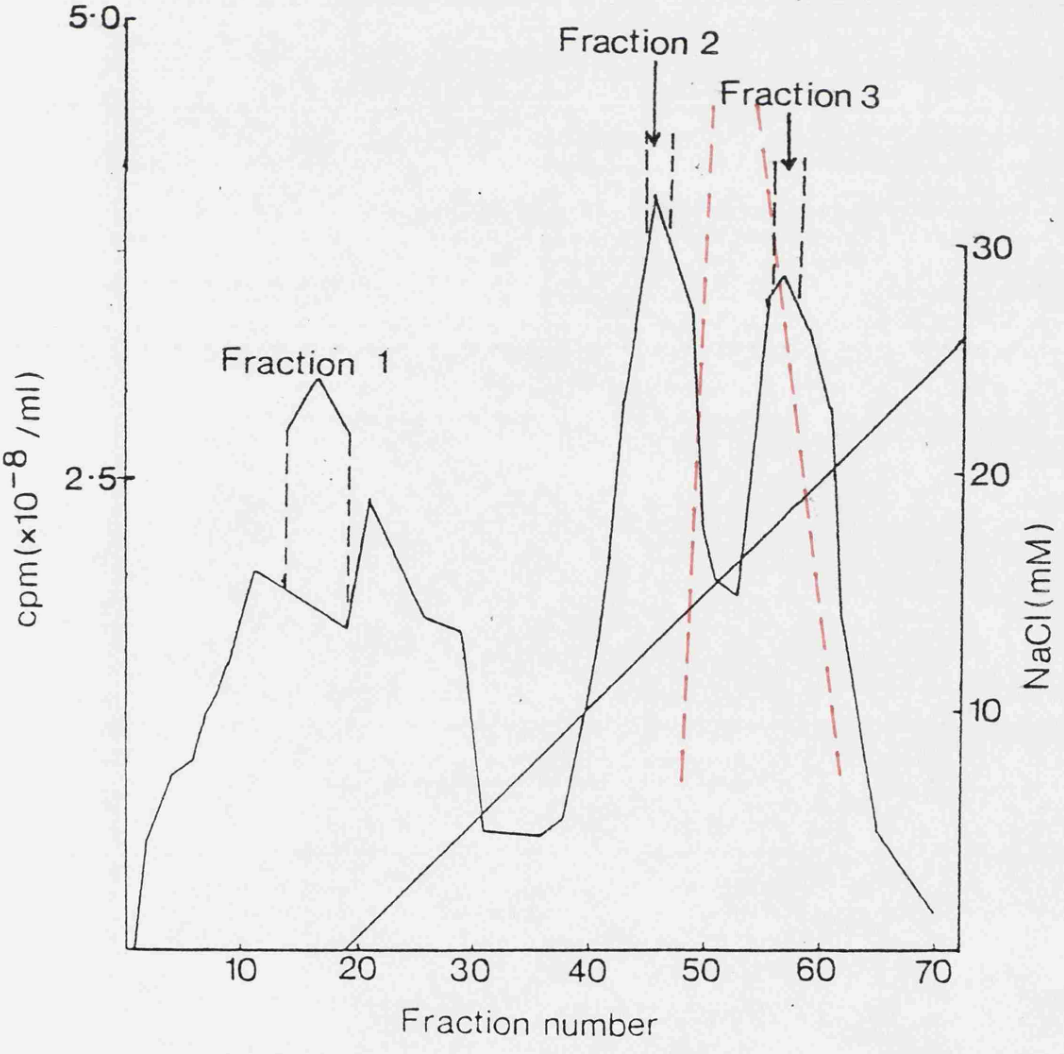


FIGURE 23b. Fractionation of ^{125}I-α- bungarotoxin (100μg) on CM - Sephadex C50.

The dashed line shows the probable elution position of unlabelled toxin. - - -



AChR bound a maximum of 68% of fraction 2, and 80% of fraction 3, while 53% of an aliquot of the ^{125}I -α-BGT taken prior to fractionation was bound to the receptor.

Co-chromatography of unlabelled α-BGT and mono-iodo α-BGT from CM-Sephadex has been reported (Blanchard et al., 1979), and therefore an experiment was designed to determine whether either fraction 2 or 3 also contained unlabelled toxin. This was done by determining the concentration of a preparation of Torpedo AChR by binding of serial dilutions of AChR to unfractionated ^{125}I -α-BGT, or mono -, or di-iodinated α-BGT. Table 14 shows the results obtained, basing the receptor concentration calculation on the concentration of mono - or di-iodo toxin previously calculated. The results show that mono-iodo α-BGT seriously underestimated the AChR concentration, compared with both unfractionated and di-iodo toxin, implying the presence of unlabelled α-BGT co-chromatographing with this fraction in an approximately equal concentration with the labelled toxin. The probable elution position of unlabelled α-BGT from the ion-exchange column is, therefore, also shown in Figure 23b. To confirm further that the di-iodo α-BGT fraction was free of unlabelled toxin, the di-iodo toxin was mixed with a theoretically equal amount of unlabelled toxin, and this mixture was then bound to the receptor. Under these conditions, double the amount of AChR was required to bind the same percentage of excess α-BGT from the toxin mixture than with the pure di-iodo fraction, indicating again that the di-iodo toxin contained no unlabelled α-BGT.

The saturation binding of di-iodo α-BGT to AChR on chick myotube cultures was also compared with that of unfractionated ^{125}I -α-BGT. A mean of 93 fmol per culture dish of unfractionated toxin

TABLE 14. Torpedo AChR concentration calculated by binding to mono- and di-iodinated α- bungarotoxin.

α- bungarotoxin preparation	AChR concentration (pmol/ml)
Unfractionated ^{125}I-α-BGT	6588
Mono-iodo ^{125}I-α-BGT	3112
Di-iodo ^{125}I-α-BGT	6355

BGT ; bungarotoxin.

(n = 3 experiments on triplicate cultures) were bound to the myotubes, compared with 92 fmol of di-iodo α-BGT (n = 3).

^{125}I - α-BGT binding to AChR

A typical binding curve of ^{125}I - α-BGT binding to purified Torpedo AChR is shown in Figure 24. AChR concentration was determined from the linear portion of the binding curve, where toxin was in excess over receptor. The calculation of AChR concentration assumed total recovery of α-BGT from the iodination procedure. No correction for the biological activity of the ^{125}I - α-BGT has been made in the calculation of AChR concentrations quoted in this thesis.

The specific binding of ^{125}I - α-BGT to chick myotube cultures is shown in Figure 25. The K_D for the binding was calculated, from the ^{125}I - α-BGT concentration giving half maximal binding, to be approximately 2.5 x 10^{-9} M. For routine determinations of ^{125}I - α-BGT binding capacity of myotube cultures, the toxin was used at a final concentration in excess of 10 nM.

A typical concentration curve for the specific binding of ^{125}I - α-BGT to human myotubes is shown in Figure 26. The binding at saturation was much less than that to chick myotube cultures. The K_D was calculated to be 6.5 x 10^{-9} M. Of note also was the high concentration of decamethonium bromide (10^{-3} M) necessary to prevent α-BGT binding, and allow a measure of non-specific toxin binding.

Inhibition of ^{125}I - α-BGT binding to chick muscle AChR by Concanavalin A

Concanavalin A inhibited ^{125}I - α-BGT binding to chick myotubes by a maximum of 63% at concentrations of up to 1 mg/ml (Figure 27).

FIGURE 24. Titration of ^{125}I-α- bungarotoxin with AChR purified from Torpedo marmorata.

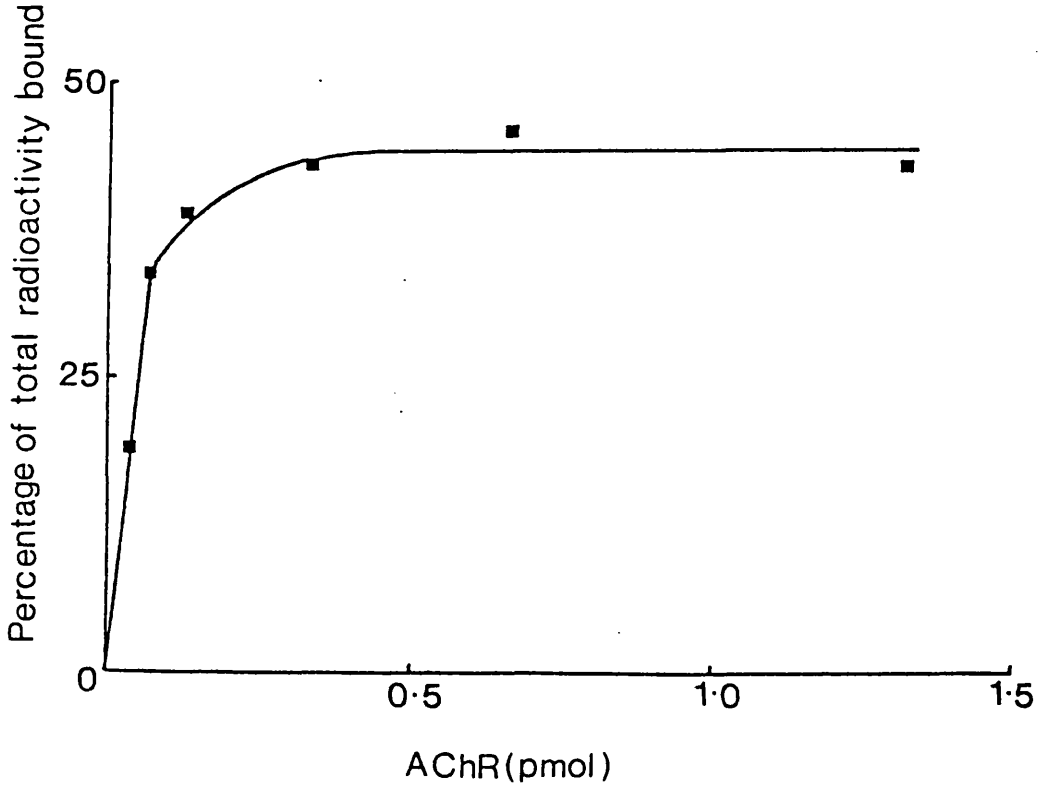


FIGURE 25. The specific binding of ^{125}I-α- bungarotoxin (BGT) to chick embryonic myotubes.

Each point represents the mean ± s.e.m. of three separate determinations on triplicate cultures of myotubes at 6-8 days in culture.

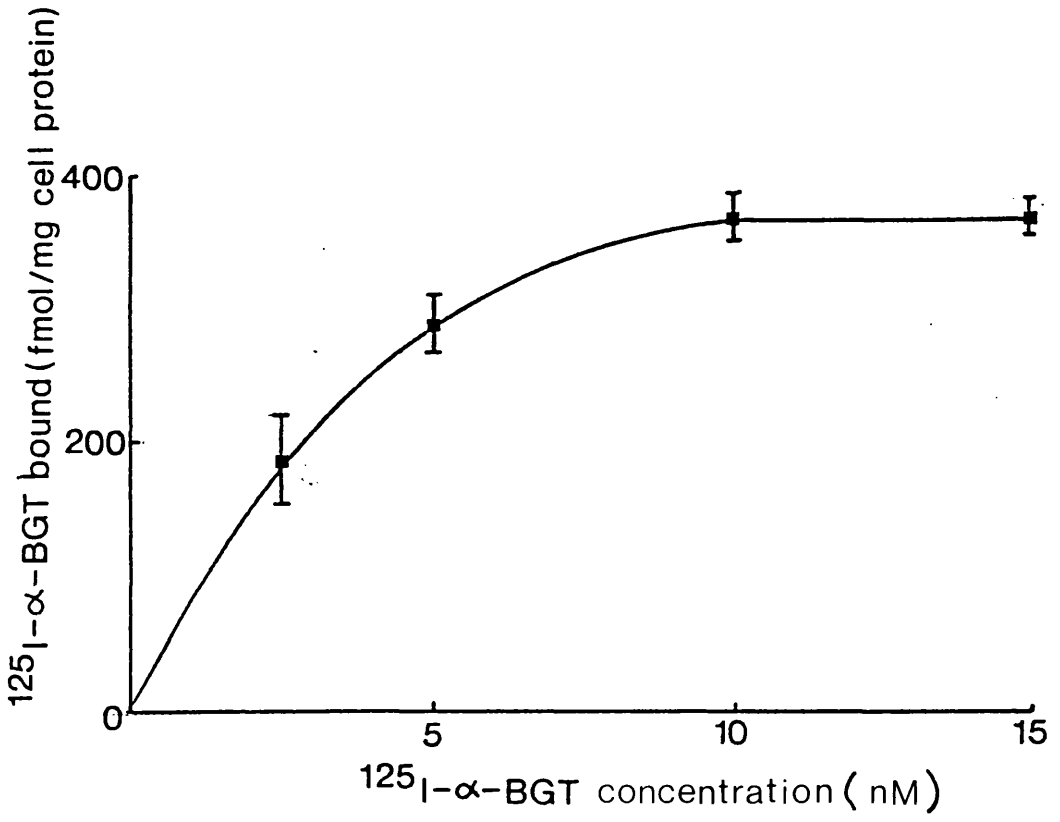


FIGURE 26. The specific binding of ^{125}I-α- bungarotoxin (BGT) to human foetal muscle cells in culture.

Each point represents the mean of two separate determinations on duplicate cultures of myotubes grown for 14 days in 199 medium, containing 10% donor horse serum.

The amount of α-BGT bound at saturation was very similar to that shown by Franklin et al (1980) for adult human muscle in culture (approximately 65f mol/mg protein).

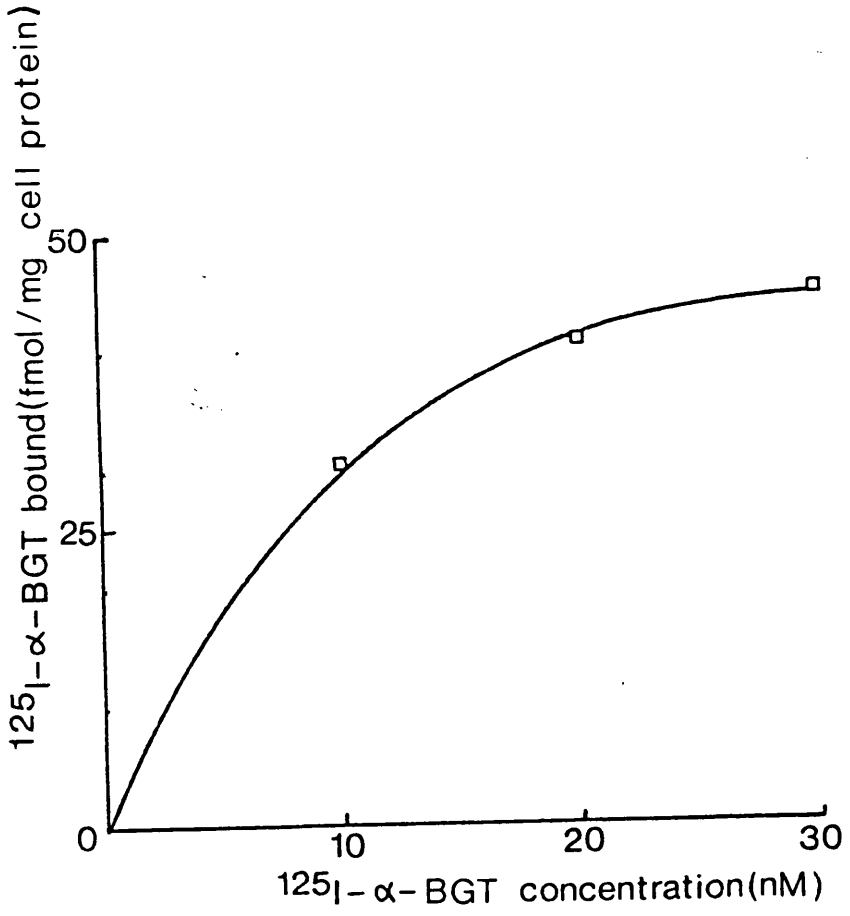
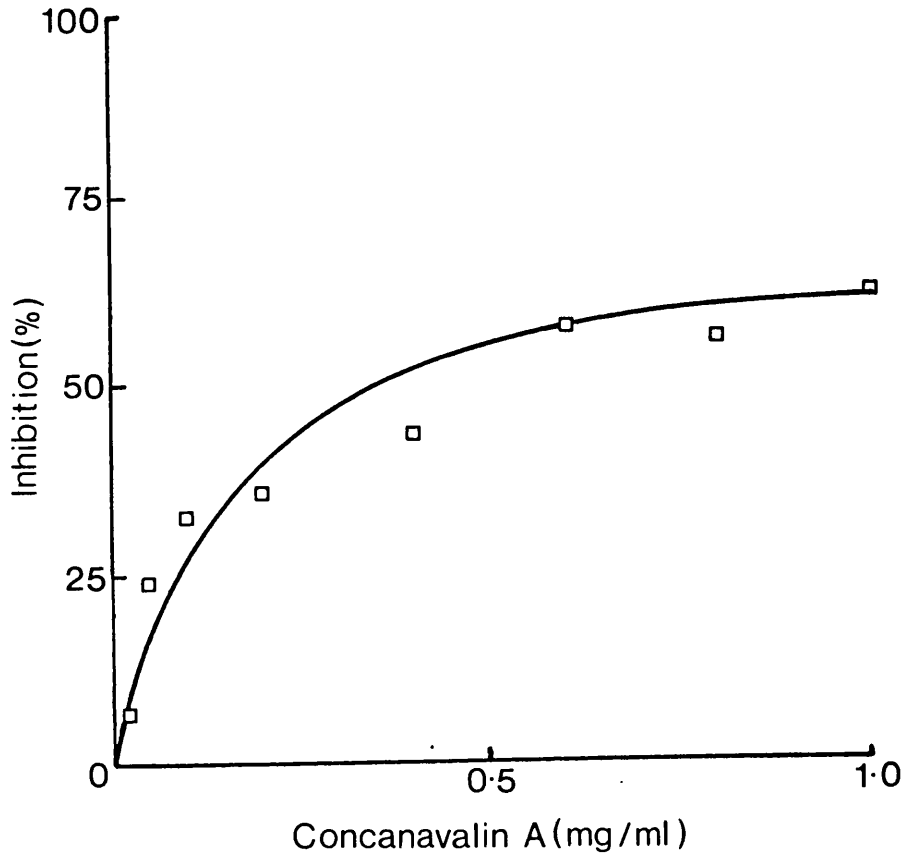


FIGURE 27. Inhibition by Concanavalin A of the specific binding of ^{125}I-α- bungarotoxin to chick embryonic myotubes.



Preparation of $F(ab')_2$ and Fab fragments

The titre of anti - (Torpedo AChR) antibody in the sheep antiserum used in this thesis was approximately 30 μM (expressed as moles ^{125}I - α - BGT binding sites precipitated per litre of serum). The antiserum gave a single precipitin line in immunodiffusion and rocket electrophoresis with purified AChR from Torpedo marmorata (Barkas et al., 1978).

$F(ab')_2$ were prepared from IgG by peptic digestion, and separated from undigested IgG, Fab fragments and smaller peptides by gel filtration on Ultrogel ACA 34. Figure 28 shows a typical elution profile of pepsin - digested IgG from ACA 34. The profile was similar for non-immune or immune IgG, with the relative proportions of $F(ab')_2$ and Fab varying from preparation to preparation. $F(ab')_2$ was, however, always predominant. A relatively pure preparation of $F(ab')_2$ was achieved by collecting only the central portion of the $F(ab')_2$ peak (Figure 28).

The purity of $F(ab')_2$ and Fab fragments was assessed by SDS polyacrylamide gel electrophoresis under non-reducing conditions. A typical gel profile is shown in Figure 29. Both immune and non-immune Fab (slots 1 and 2) showed a single major band at 50000 daltons. Immune and non-immune $F(ab')_2$ (slots 3 and 4) ran as a single major band at 100000 daltons.

Affinity purification of $F(ab')_2$ fragments

The affinity column used for purification of anti-AChR $F(ab')_2$ fragments consisted of Torpedo AChR bound to α-BGT, which was itself covalently attached to a Sepharose 4 B solid support. This avoided any distortion or shielding of antigenic sites on the receptor which

FIGURE 28. Gel filtration of pepsin digested sheep IgG on Ultrogel ACA 34.

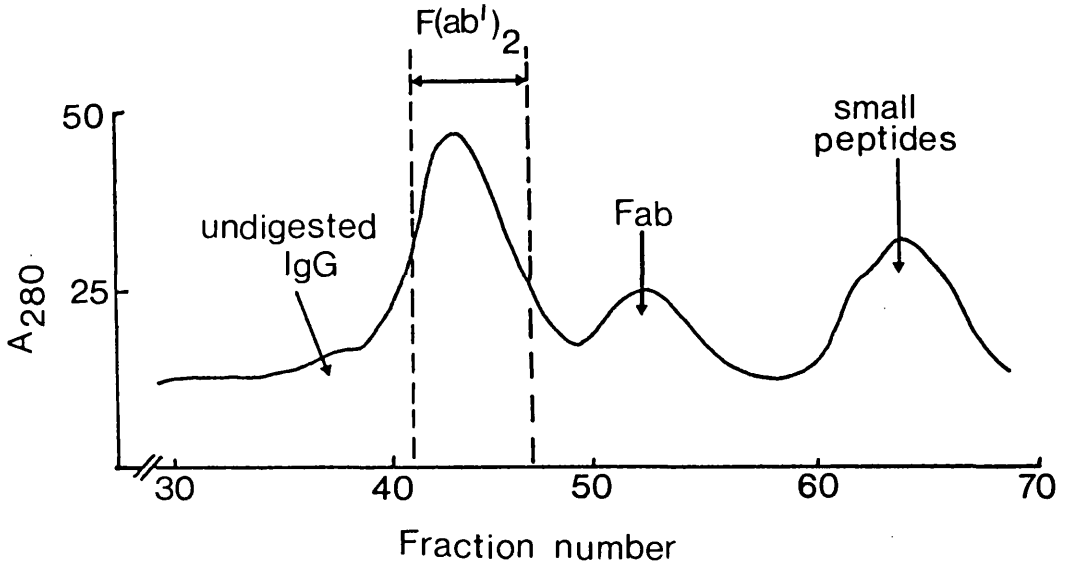


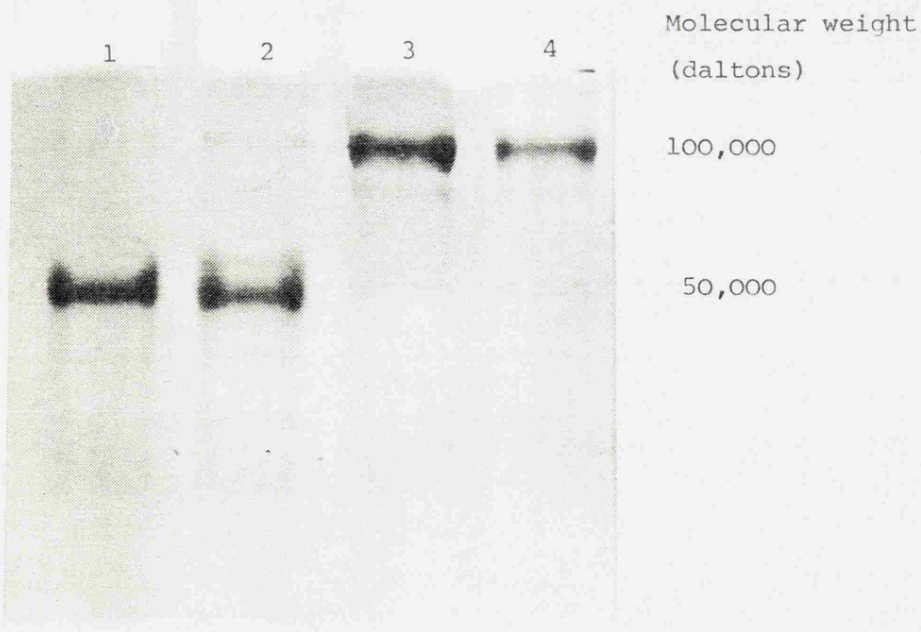
FIGURE 29. An SDS polyacrylamide electrophoresis gel of Fab and $F(ab')_2$ fragments from sheep IgG.

Track 1. Immune Fab

Track 2. Non-immune Fab

Track 3. Immune $F(ab')_2$

Track 4. Non-immune $F(ab')_2$



might arise from covalent coupling of the AChR to Sepharose 4B (Schwartz et al., 1979). After incubation of the affinity gel with immune $F(ab')_2$, most of the $F(ab')_2$ preparation was eluted in the void volume (non-bound fraction). A consistent 5% of the $F(ab')_2$ fragments remained bound to the column. These were considered to be the specific anti-Torpedo AChR antibody fragments within the immune $F(ab')_2$ preparation, and a value of 5% of the total $F(ab')_2$ concentration was consequently used in calculating the "free $F(ab')_2$" concentration in the Scatchard analysis of specific $F(ab')_2$ binding to both Torpedo AChR and chick myotube AChR (Figures 38 and 42) and the binding analysis of the inhibition of α-BGT binding by $F(ab')_2$ fragments (Figure 35). The affinity-bound $F(ab^1)_2$ Fragments were eluted from the column with 0.2 M ammonium hydroxide, following the method of Martinez et al (1977). These elution conditions were chosen instead of the more conventionally used acidic conditions (Hudson and Hay, 1976) because of the sensitivity of Torpedo AChR to acid pH (Saitoh et al., 1979; Wonnacott et al., 1980a). The ammonium hydroxide-eluted $F(ab')_2$ was quickly brought to neutral pH by gel filtration in order to prevent denaturation.

Preparation of ^{125}I - $F(ab')_2$ and Fab fragments

Early attempts to radio-iodinate $F(ab')_2$ fragments were performed in 0.1 M Tris buffer, pH 8.0 containing 0.15 M NaCl, and produced the type of elution profile from Sephadex G 50 shown in Figure 30. The incorporation of ^{125}I into protein averaged only 34% (n = 2).

In an attempt to increase this incorporation $F(ab')_2$ was transferred into 0.1 M phosphate buffer, pH 7.0 by dialysis prior to the iodination reaction. The elution profiles of immune and non-immune $F(ab')_2$ from Sephadex G 50 following iodination under these conditions are shown in Figure 31 a and b. Immune $F(ab')_2$ was labelled to a specific activity of 27 ± 0.6 Ci/mmol (n = 10), with 81 ± 2% (n = 10) incorporation into protein of the total ^{125}I added to the reaction vessel. Non-immune ^{125}I - $F(ab')_2$ had a specific activity of 28 ± 0.3 Ci/mmol (n = 10), with 84 ± 0.9% (n = 10) incorporation of ^{125}I into protein.

Typical elution profiles from Sephadex G 50 for immune and non-

FIGURE 30. Gel filtration of immune ^{125}I-F(ab')$_2$ on Sephadex G50 at pH 8.

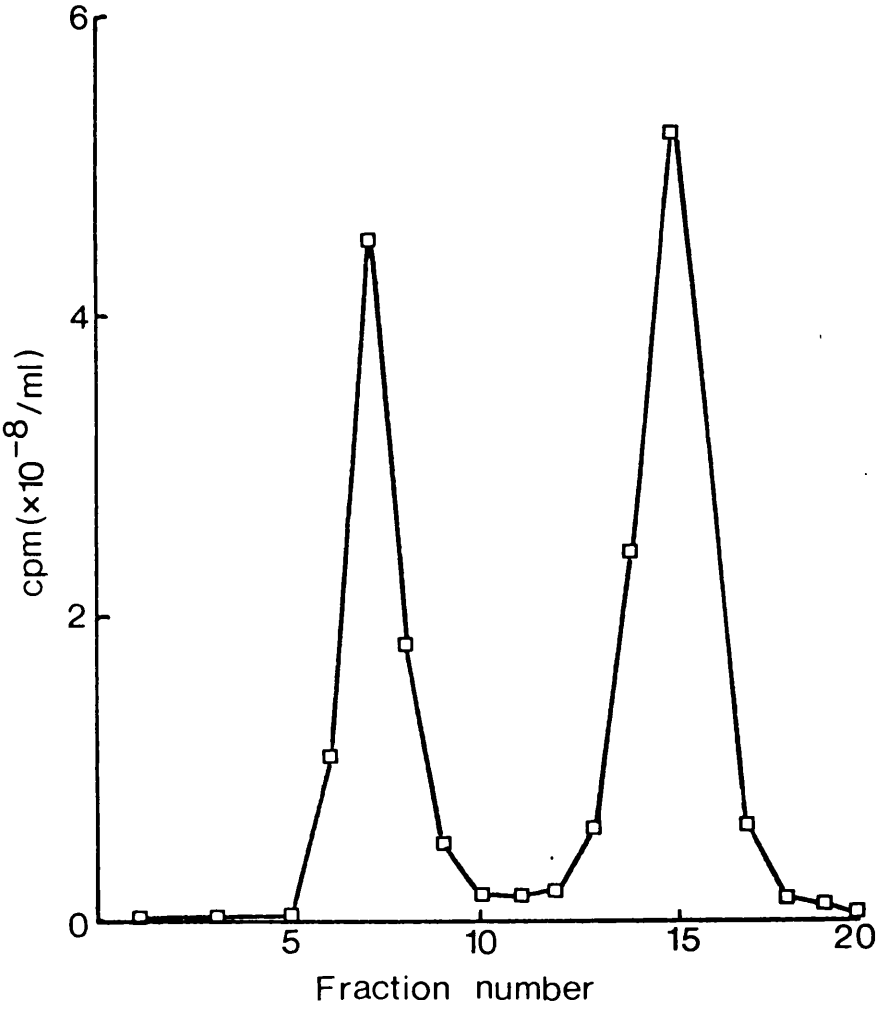
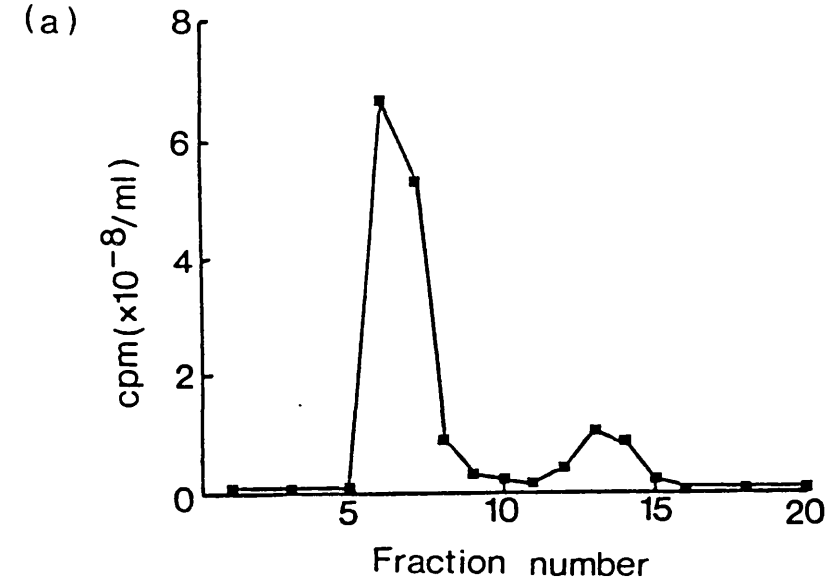


FIGURE 31. Gel filtration of ^{125}I-F(ab')$_2$ on Sephadex G50 at pH 7.

(a) Immune ^{125}I-F(ab')$_2$.

(b) Non-immune ^{125}I-F(ab')$_2$.



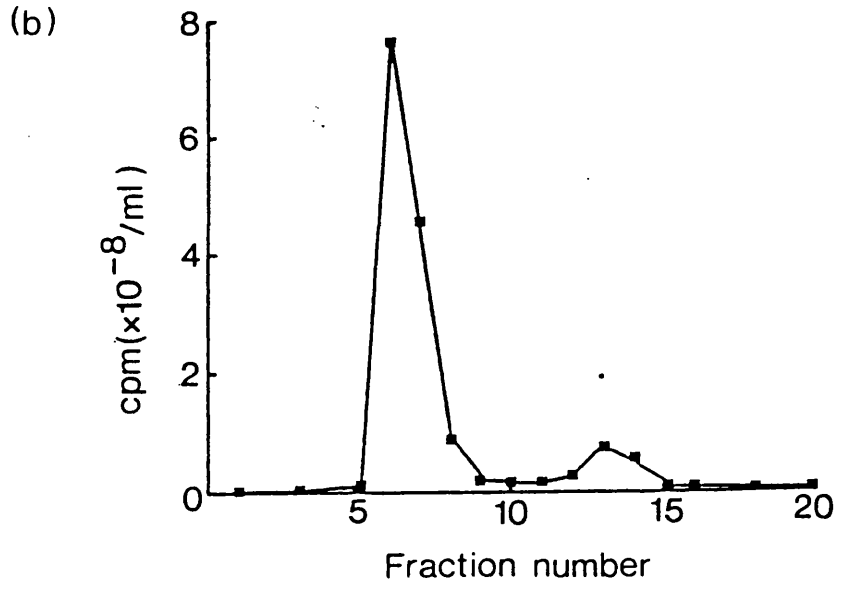
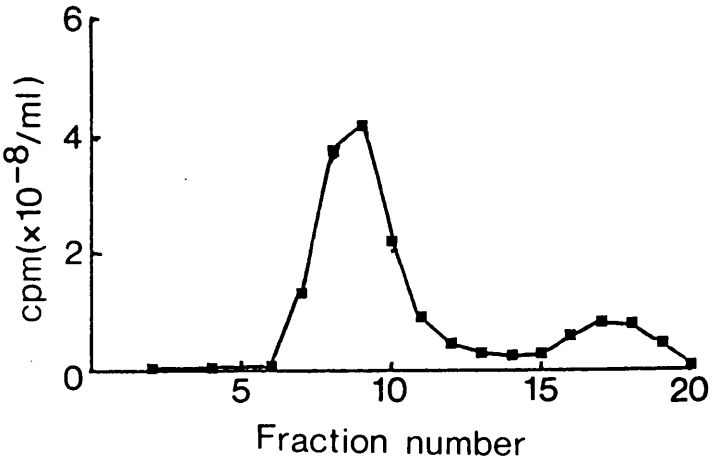
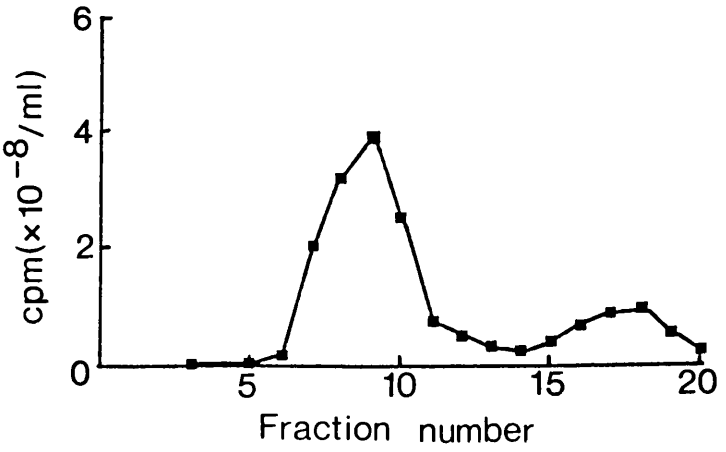


FIGURE 32. Gel filtration of ^{125}I-Fab on Sephadex G50.

(a) Immune ^{125}I-Fab

(b) Non-immune ^{125}I-Fab





immune Fab fragments, iodinated in 0.1 M phosphate buffer, pH 7.0, are shown in Figure 32 a and b. The protein peak was generally broader than that obtained with $F(ab')_2$. Immune Fab was labelled to a specific activity of 13.6 $\pm$ 0.1 Ci/mmol (n = 3), with 81 $\pm$ 1.2% incorporation of ^{125}I into protein. Non-immune Fab was labelled to a specific activity of 14.0 $\pm$ 0.1 Ci/ mmol (n = 3), with 84 $\pm$ 0.6% (n = 3) incorporation of ^{125}I into protein.

Inhibition of ^{125}I -α-BGT binding to AChR by $F(ab')_2$ and Fab fragments

The inhibition of binding of ^{125}I -α-BGT to purified Torpedo AChR by pre-incubation with immune or non-immune $F(ab')_2$ and Fab fragments is shown in Figure 33. The corresponding inhibition of ^{125}I -α-BGT binding to chick myotube cultures by $F(ab')_2$ and Fab fragments is shown in Figure 34. The binding of the inhibitory immune $F(ab')_2$ and Fab fragments in the two systems was analysed by use of the Hill equation (Figure 35). The degree of inhibition of toxin binding was used as a measure of the fractional saturation of the IgG fragments to AChR, taking Bmax to be the maximum inhibition observed. The x - axis represents $\log_{10}$ of specific anti - (Torpedo AChR) $F(ab')_2$ or Fab, and was calculated as being 5% of the total $F(ab')_2$ or Fab pre-incubated with receptor (see affinity purification of $F(ab')_2$ above). The values of n and K obtained from this analysis are given in Table 15.

AChR turnover from chick myotubes in culture

AChR turnover from chick myotubes was measured by loss of specifically bound ^{125}I -α-BGT into the incubation medium, both in the presence and absence of immune $F(ab')_2$ fragments. Figure 36 shows

FIGURE 33. The inhibition of specific ^{125}I-α- bungarotoxin binding to Torpedo marmorata AChR by Fab and F(ab')$_2$ fragments.

- □ Non-immune Fab
- ■ Immune Fab
- △ Non-immune F(ab')$_2$
- ▲ Immune F(ab')$_2$

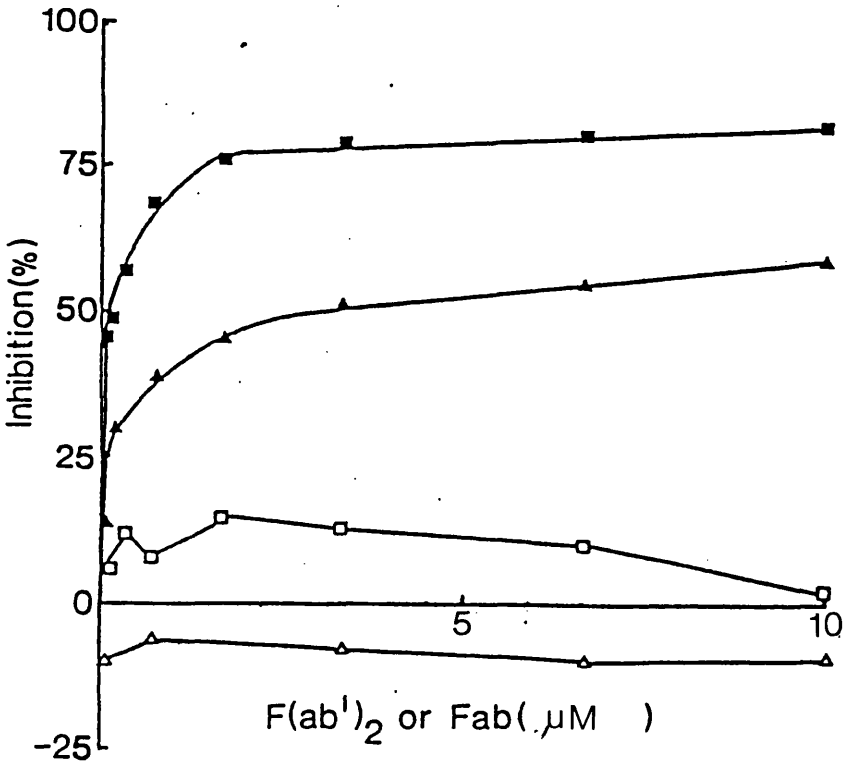


FIGURE 34. The inhibition of specific ^{125}I-α- bungarotoxin binding to chick embryonic myotube cultures by Fab and F(ab')$_2$ fragments.

△ Non-immune Fab

▲ Immune Fab

□ Non-immune F(ab')$_2$

■ Immune F(ab')$_2$

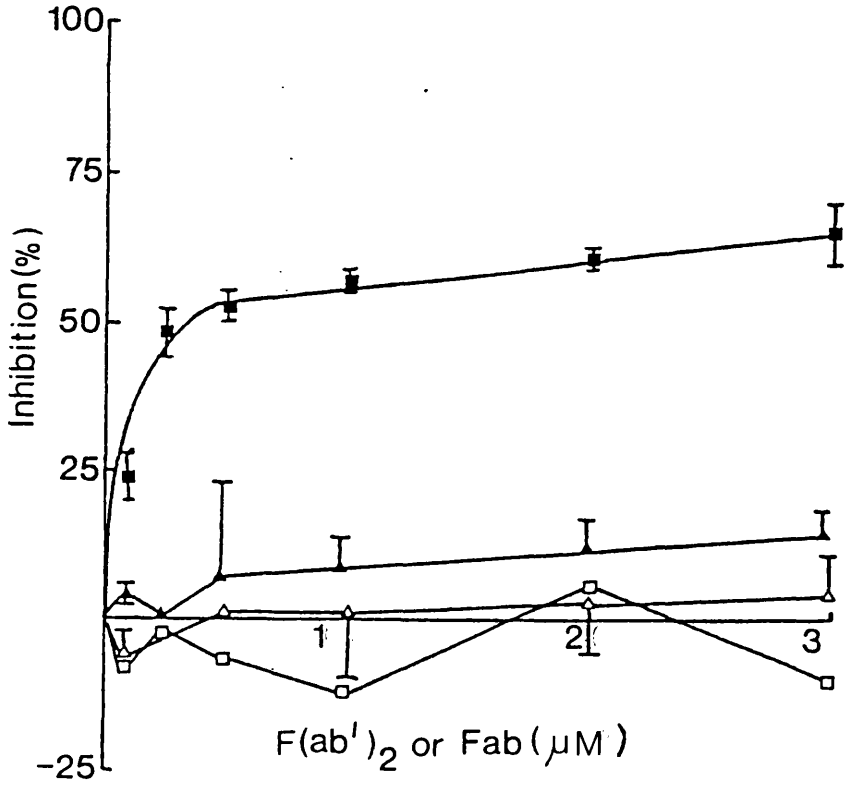


FIGURE 35. Hill plots for the inhibition of ^{125}I-α- bungarotoxin binding to AChR by immune Fab and F(ab')$_2$ fragments. shown in Figures 33 and 34.

In each case:

B = The percentage inhibition of ^{125}I-α- bungarotoxin binding

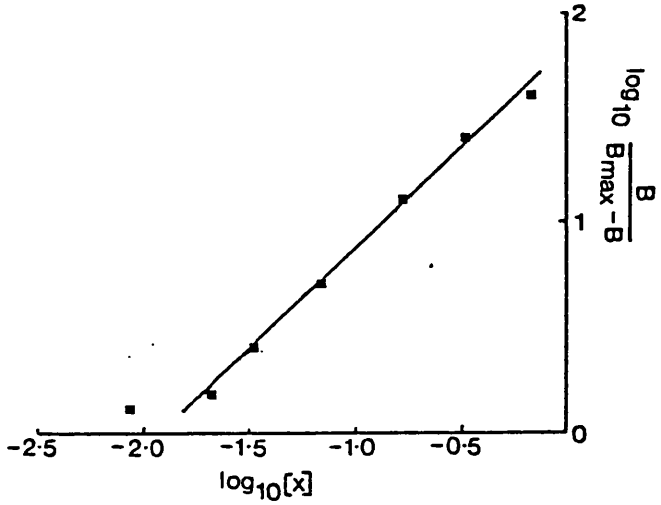
B_{max} = The maximum inhibition of ^{125}I-α- bungarotoxin binding

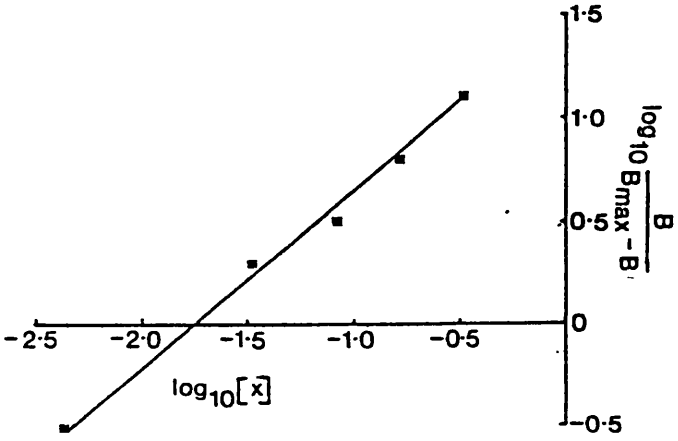
[x] = The concentration of specific anti-Torpedo AChR fragments, calculated as 5% of the total immune Fab or F(ab')$_2$ preparation added (x-axis in Figures 33 and 34), based on the percentage of immune F(ab')$_2$ which bound to an affinity column of toxin-conjugated Torpedo AChR (Results p.138)

(a) Inhibition of toxin binding to Torpedo AChR by Fab.

(b) Inhibition of toxin binding to Torpedo AChR by F(ab')$_2$.

(c) Inhibition of toxin binding to chick embryonic myotubes by F(ab')$_2$.


2
1
0
-2·5
-2·0
-1·5
-1·0
-0·5
log10 [x]
log10 B/(Bmax - B)


1·5
1·0
0·5
0
-0·5
-2·5
-2·0
-1·5
-1·0
-0·5
log10 [x]
log10 B/(Bmax - B)

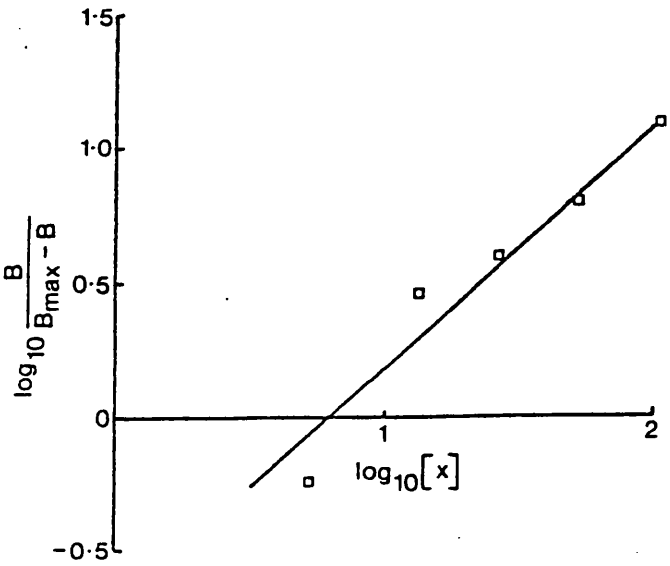

1·5
1·0
0·5
0
-0·5
1
2
log10 [x]
log10 B/(Bmax - B)

TABLE 15. Values of the dissociation constant (K_D) and Hill coefficient (n) for the α- bungarotoxin blocking population of antibody fragments derived from sheep anti-AChR antiserum.

Antibody fragment	Antigen	K_D	n
Fab	Purified Torpedo AChR	1.4×10^{-8}M	1
$F(ab')_2$	Purified Torpedo AChR	3×10^{-8}M	0.85
$F(ab')_2$	Chick myotube AChR	5×10^{-9}M	0.9

The values of K_D and n were calculated from the Hill plots shown in Figure 35, by use of the Hill equation (Methods p 90)

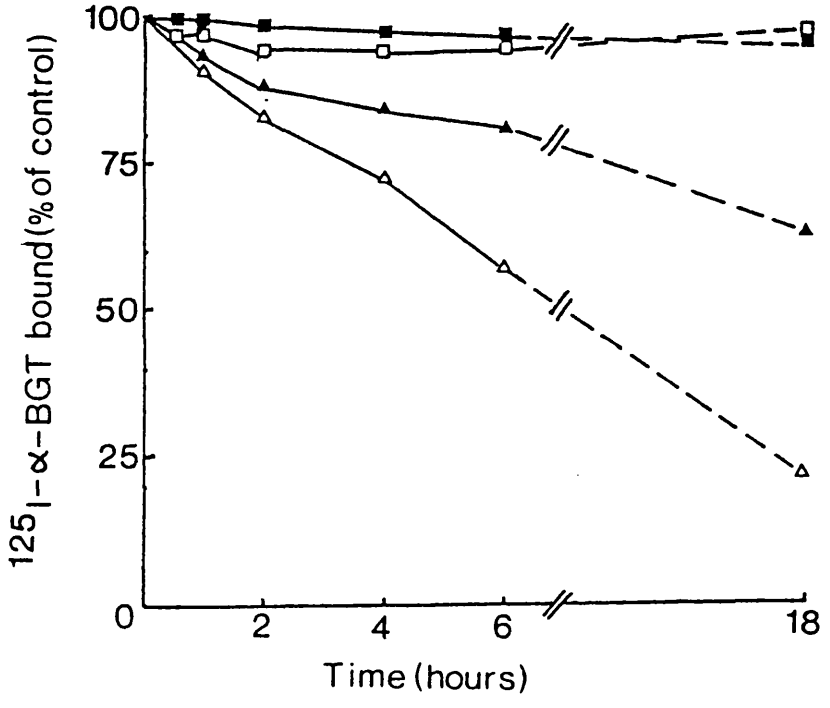
FIGURE 36. Effects of immune $F(ab')_2$ on the degradation rate of the AChR in cultured chick embryonic myotubes.

■ AChR degradation in the absence of $F(ab')_2$ at 4°C.

□ AChR degradation in the presence of $F(ab')_2$ at 4°C.

▲ AChR degradation in the absence of $F(ab')_2$ at 37°C.

△ AChR degradation in the presence of $F(ab')_2$ at 37°C.



that at 4°C loss of bound ^{125}I -α-BGT was negligible, whether $F(ab')_2$ was present or not. AChR loss from the myotubes at 37°C in the absence of $F(ab')_2$ was much greater than at 4°C, illustrating the strict temperature dependence of AChR degradation. The presence of immune $F(ab')_2$ fragments significantly increased the loss of ^{125}I - α-BGT from the myotubes at 37°C.

^{125}I - $F(ab')_2$ binding to Torpedo AChR

^{125}I - $F(ab')_2$ binding to Torpedo AChR was measured by a DEAE - cellulose filter assay. The binding of non-immune $F(ab')_2$ was minimal (Figure 37 a and b), being always < 0.2% of the total added radioactivity. Figure 37a shows the binding of ^{125}I - $F(ab')_2$ fragments to increasing concentrations of Torpedo AChR. The bound radioactivity at saturation represented a mean of 5.6% of the total added radioactivity, and indicates that all of the anti - (Torpedo AChR) antibody fragments in the $F(ab')_2$ preparation (see affinity purification of $F(ab')_2$) were bound by an excess of AChR. The binding of increasing ^{125}I - $F(ab')_2$ to a fixed concentration of receptor was both specific and saturable (Figure 37b). Scatchard analysis of this binding curve (Figure 38) indicated apparent homogeneity in the binding affinity of ^{125}I - $F(ab')_2$ to the purified AChR.

The value of K_D was calculated to be 2.5×10^{-8}M.

The binding of ^{125}I - $F(ab')_2$ to Torpedo AChR was inhibited by up to 90% following pre-incubation of receptor with the unlabelled parent immune $F(ab')_2$ or affinity - purified immune $F(ab')_2$ fragments, while both non-immune $F(ab')_2$ fragments, and the non-bound fraction

FIGURE 37. The binding of ^{125}I-F(ab')$_2$ to Torpedo marmorata AChR.

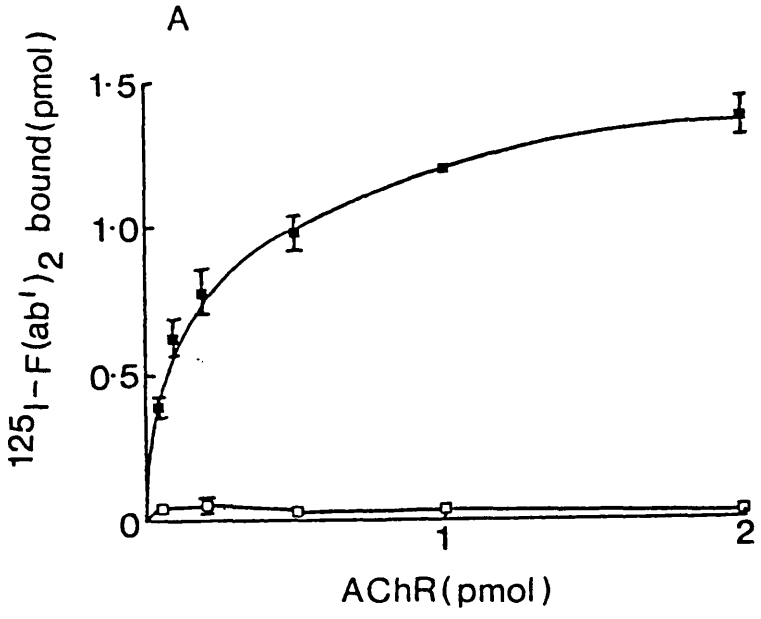
■ Immune F(ab')$_2$

□ Non-immune F(ab')$_2$

A: Binding of a fixed amount of ^{125}I-F(ab)$_2$ (25 pmol) to increasing amounts of Torpedo AChR.

B: Binding of a fixed amount of Torpedo AChR (1pmol) to increasing concentrations of ^{125}I-F(ab)$_2$

Binding was assayed by a DEAE-cellulose filter disc assay as detailed in the Methods (p89).Specific binding was taken to be the binding of immune F(ab')$_2$ fragments, since the binding of non-immune fragments was negligible. The radioactivity bound to filters from blank samples containing no AChR was subtracted from all samples as a measure of non-specific binding.


A
1·5
1·0
0·5
0
1
2
^{125}I-F(ab')$_2$ bound(pmol)
AChR(pmol)

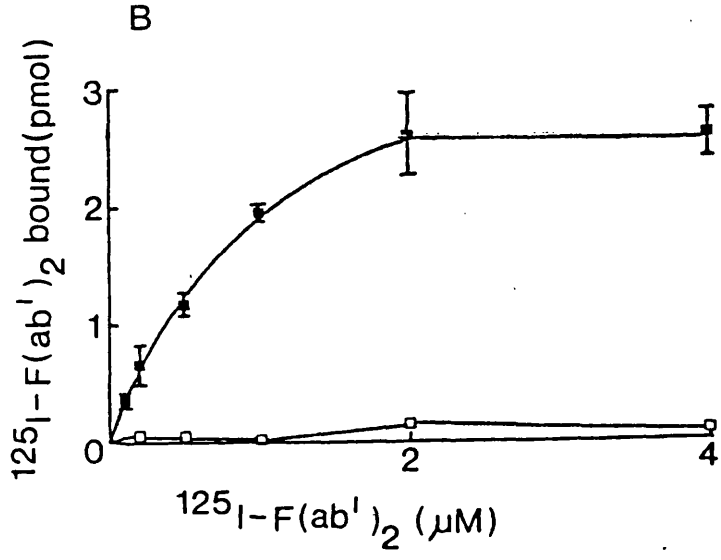

B
3
2
1
0
2
4
^{125}I-F(ab')$_2$ bound(pmol)
^{125}I-F(ab')$_2$ (μM)

FIGURE 38. Scatchard analysis of the specific binding of anti-Torpedo AChR ^{125}I-F(ab')$_2$ to purified Torpedo marmorata AChR.

The concentration of free ^{125}I-F(ab')$_2$ was that of specific anti-Torpedo AChR F(ab')$_2$, calculated as 5% of the total concentration of the immune ^{125}I-F(ab')$_2$ preparation added (x-axis in Figure 37b), based on the percentage of immune F(ab')$_2$ which bound to an affinity column of toxin-conjugated Torpedo AChR (Results p.138).

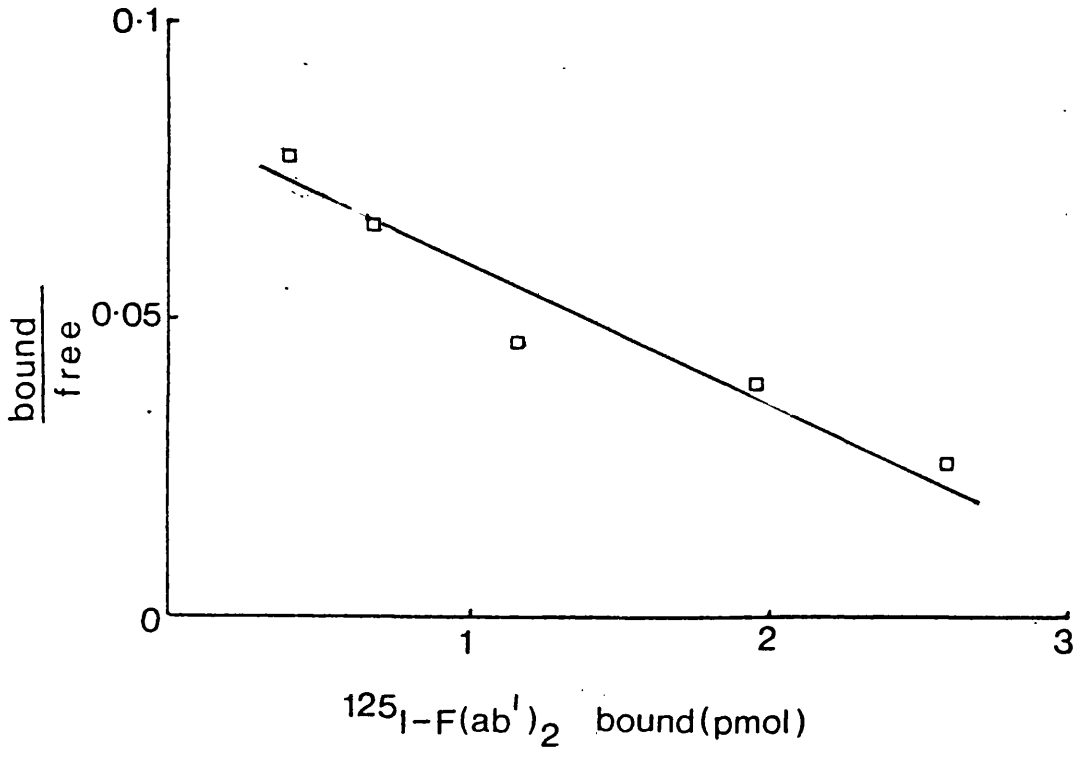


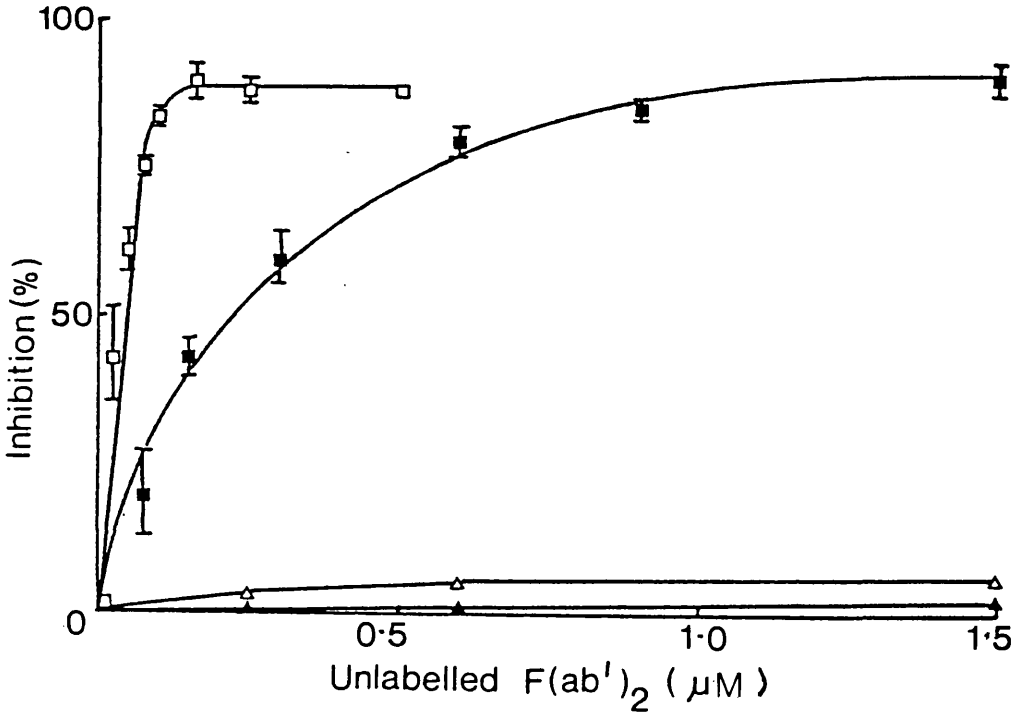
FIGURE 40. Inhibition of the specific binding of ^{125}I-F(ab')$_2$ fragments to Torpedo marmorata AChR by unlabelled F(ab')$_2$ fractions.

▲ Non-immune F(ab')$_2$ fragments

■ Parent immune F(ab')$_2$ fragments

△ Nonbound fraction from toxin-AChR affinity column

□ Affinity purified F(ab')$_2$ fragments



from the toxin-receptor affinity column were without effect (Figure 40).

^{125}I - $F(ab')_2$ binding to chick myotubes in culture

The binding of immune and non-immune ^{125}I - $F(ab')_2$ fragments to chick embryo myotubes is shown in Figure 41. The binding of non-immune $F(ab')_2$ was much greater in this system than that to purified Torpedo AChR, probably due to non-specific, or low affinity binding to membrane antigens on the surface of the myotubes, other than AChR. Specific binding of anti-AChR $F(ab')_2$ was taken to be the difference between binding of immune and non-immune $F(ab')_2$. This specific binding was subjected to Scatchard analysis (Figure 42). Although the number of data points is small, the plot indicates a relative homogeneity of binding affinity. K_D was calculated to be 1×10^{-7} M.

^{125}I - Fab binding to chick myotube cultures

The binding of immune and non-immune ^{125}I - Fab fragments to myotube cultures is shown in Figure 43. The binding of ^{125}I - Fab to cultures was much more variable than that of ^{125}I - $F(ab')_2$, with saturable binding not always being achieved. Specific binding of ^{125}I - Fab was taken to be the difference in binding between immune and non-immune ^{125}I - Fab.

Inhibition of ^{125}I - $F(ab')_2$ binding to AChR by α-BGT

Figure 45 shows that pre-incubation of purified Torpedo AChR with an excess of unlabelled α-BGT prior to ^{125}I - $F(ab')_2$ binding reduced the specific binding of immune ^{125}I - $F(ab')_2$ by an average of only 3.5%.

FIGURE 41. The binding of ^{125}I-F(ab')$_2$ to chick embryonic myotubes.

■ Immune ^{125}I-F(ab')$_2$

□ Non-immune ^{125}I-F(ab')$_2$

The dashed line indicates the specific binding of anti-AChR ^{125}I-F(ab')$_2$, calculated as the difference between the binding of immune and non-immune ^{125}I-F(ab')$_2$.

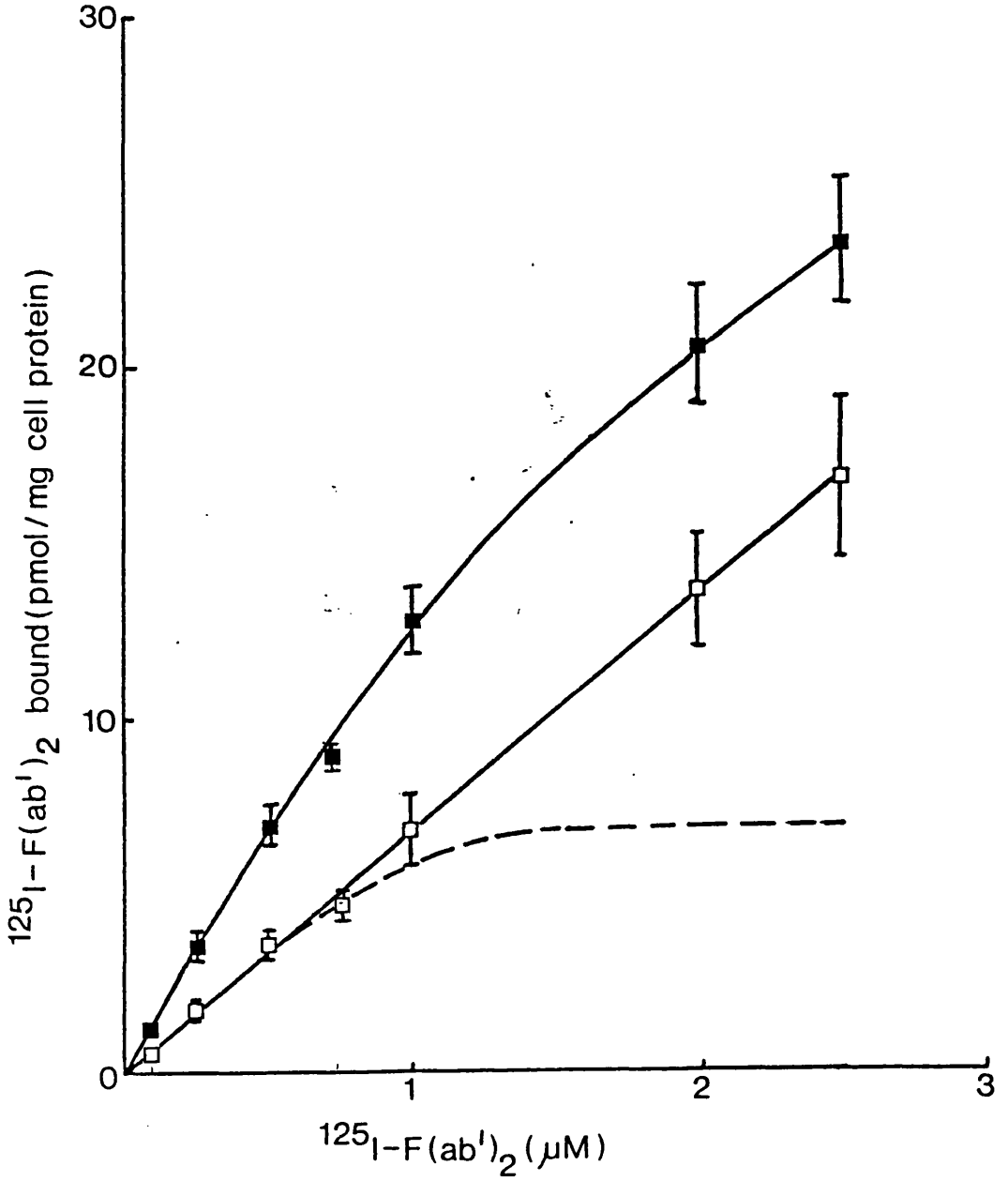


FIGURE 42. Scatchard analysis of the specific binding of anti-Torpedo AChR ^{125}I-F(ab')$_2$ to chick embryonic myotubes.

The concentration of free ^{125}I-F(ab')$_2$ is that of specific anti-receptor F(ab')$_2$, calculated as 5% of the total concentration of the immune ^{125}I-F(ab')2 added (x-axis in Figure 41), based on the percentage of immune F(ab')$_2$ which bound to an affinity column of toxin-conjugated Torpedo AChR (Results p. 138).

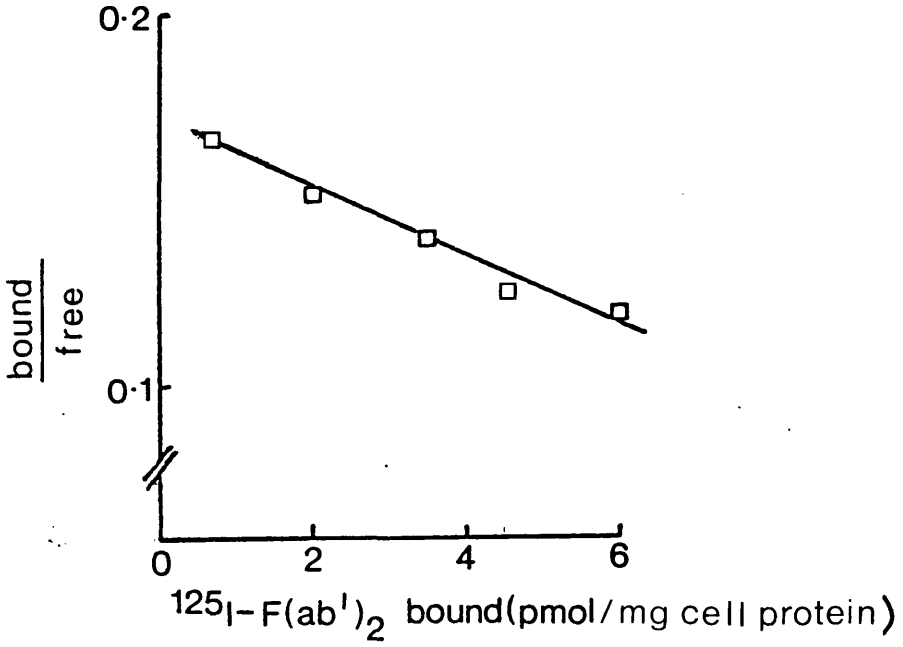


FIGURE 43. The binding of ^{125}I-Fab fragments to chick embryonic myotubes.

□ Non-immune ^{125}I-Fab

■ Immune ^{125}I-Fab

The dashed line indicates the specific binding of anti-AChR Fab calculated as the difference between the binding of immune and non-immune ^{125}I-Fab fragments.

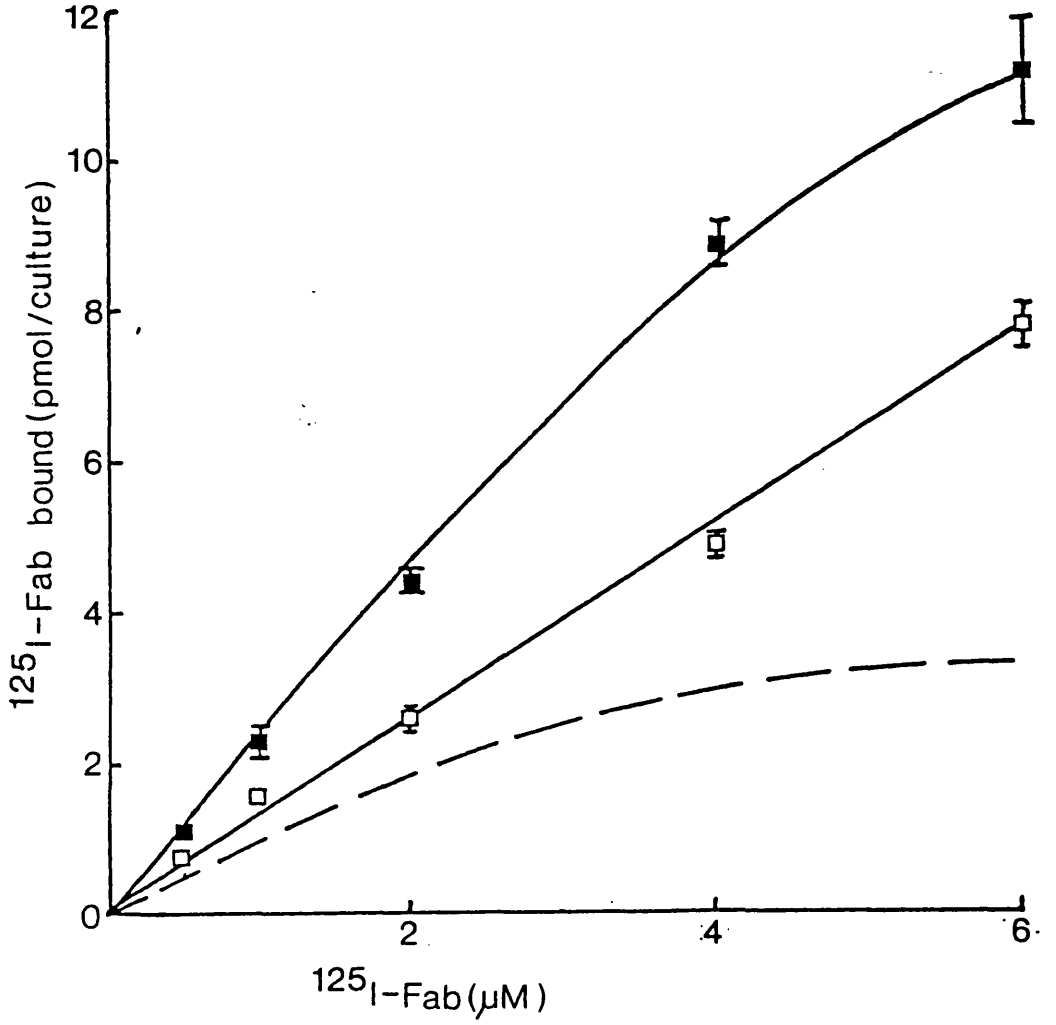


FIGURE 45. The effect of α- bungarotoxin on the specific binding of ^{125}I-F(ab')$_2$ to Torpedo marmorata.

■ Binding in the absence of α- bungarotoxin.

□ Binding in the presence of α- bungarotoxin (100 pmol)

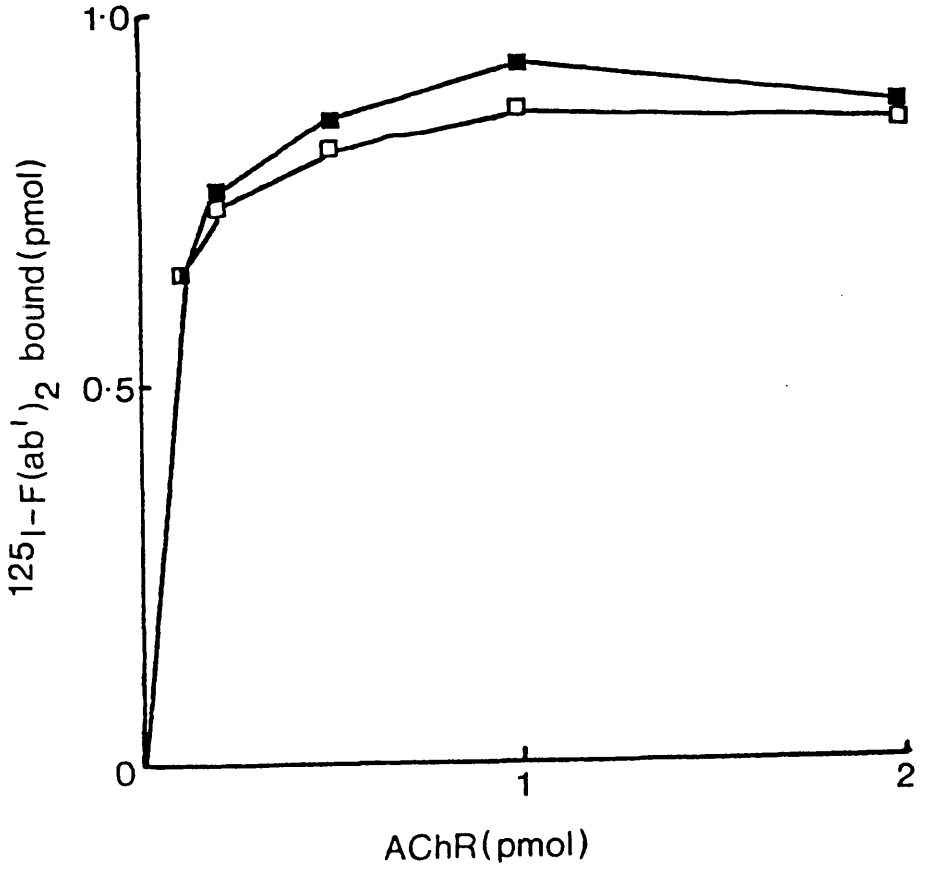


TABLE 16. Inhibition of specific ^{125}I-F(ab')$_2$ binding to chick embryonic myotubes by α- bungarotoxin.

^{125}I-F(ab')$_2$ (pmol/dish)	α- bungarotoxin (nM)	Inhibition (%)
1.0	0	0
	10	5
	20	0
2.0	0	0
	10	12
	20	-4

Specific ^{125}I - $F(ab')_2$ binding to AChR on chick myotube cultures was likewise unaffected by pre-incubation of cultures with excess α-BGT (Table 16), with essentially no inhibition of binding at either subsaturating (1 nM) or saturating (2 nM) concentrations of ^{125}I - $F(ab')_2$.

Inhibition of ^{125}I - $F(ab')_2$ binding to chick myotube cultures by Torpedo AChR

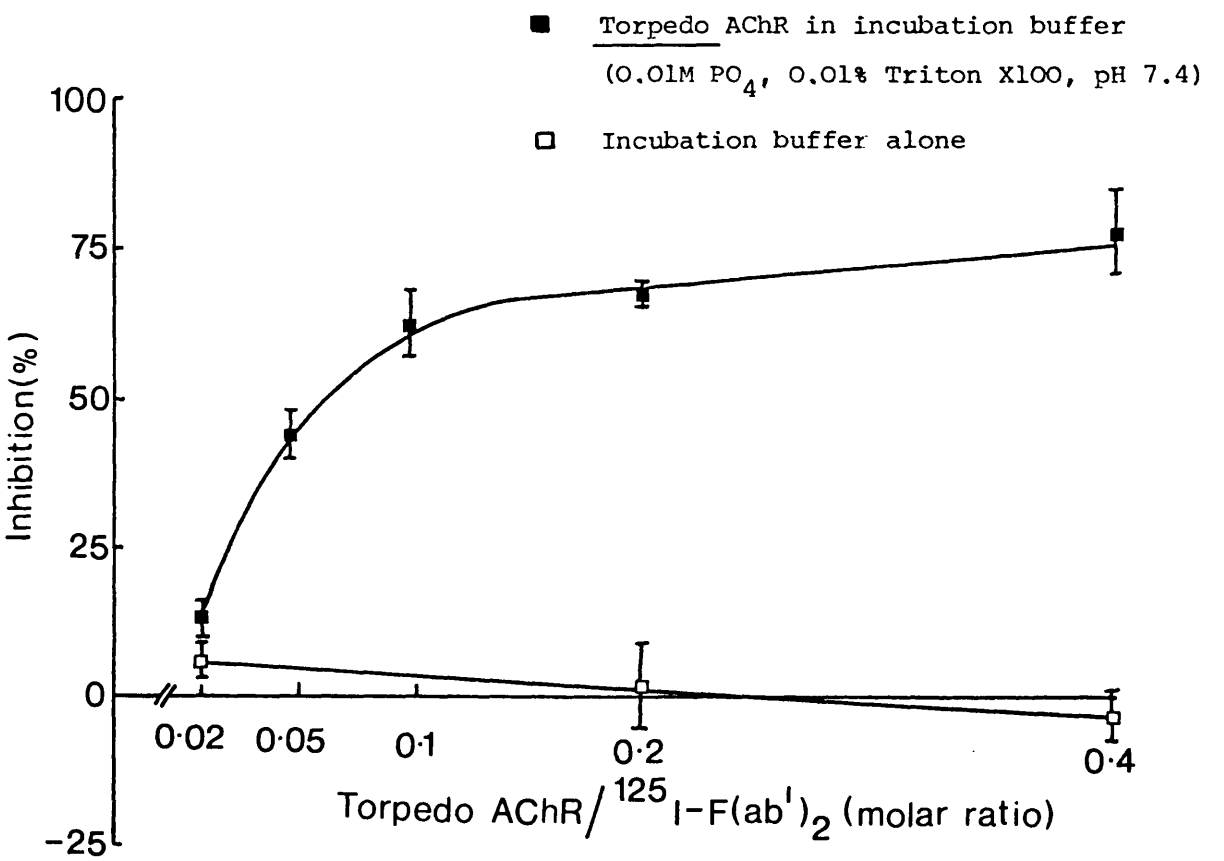
Pre-incubation of ^{125}I - $F(ab')_2$ with purified Torpedo AChR reduced the specific binding of ^{125}I - $F(ab')_2$ to chick myotubes by up to approximately 80% (Figure 46). The binding of ^{125}I - $F(ab')_2$ after pre-incubation with 10 mM phosphate buffer, pH 7.4 containing 0.1% (v/v) Triton X100 was also determined. The final concentration of Triton X100 in the binding assay was always less than 0.01%, and this concentration had no significant effect on ^{125}I - $F(ab')_2$ binding to the cultures (Figure 46). Microscopic examination of the myotube cultures after exposure to 0.01% (v/v) Triton X100 showed that no gross morphological damage to the myotubes had occurred during the binding procedure.

Effect of heat - inactivation on ^{125}I - $F(ab')_2$ binding to AChR

The α-BGT binding capacity of Torpedo AChR was almost totally abolished, with only 0.4% of the initial toxin binding capacity remaining, after 10 min exposure of the AChR at 60°C. In contrast the specific binding of ^{125}I - $F(ab')_2$ to purified Torpedo AChR was only reduced by approximately 40% after 30 min at 60°C (Figure 47).

The ability of Torpedo AChR exposed to a temperature of 60°C for 30 min to inhibit specific ^{125}I - $F(ab')_2$ binding to chick myotubes was reduced by almost 70% compared with the inhibition caused by

FIGURE 46. Inhibition of the specific binding of ^{125}I-F(ab')$_2$ fragments to chick embryonic myotubes by Torpedo marmorata AChR.



untreated receptor (Figure 47).

Effect of SDS - denaturation on ^{125}I - $F(ab')_2$ binding to AChR

The specific binding of untreated (control) and SDS - denatured Torpedo AChR to ^{125}I - $F(ab')_2$ is shown in Figure 48. The SDS - treated receptor bound approximately 35% as much immune ^{125}I - $F(ab')_2$ at saturation as control AChR.

Pre-incubation of SDS - treated Torpedo AChR with ^{125}I - $F(ab')_2$ caused only 11% inhibition of specific $F(ab')_2$ binding to chick myotube cultures. Untreated AChR was pre-incubated with ^{125}I - $F(ab')_2$ in the presence of 0.05% (w/v) SDS (the residual SDS concentration in the denatured AChR preparation used) as a control, and caused an inhibition of ^{125}I - $F(ab')_2$ binding to the cultures of 67%. Thus the residual antigenic activity of the denatured AChR was approximately 16% of that for the untreated receptor.

Effect of periodate - oxidation on AChR binding to ^{125}I - $F(ab')_2$

The binding of periodate - treated Torpedo AChR to ^{125}I - $F(ab')_2$ was essentially the same as that of untreated receptor, with the specific binding at saturation of the modified AChR being approximately 94% of the control (Figure 49).

The effect of periodate - oxidation on the ability of Torpedo AChR to inhibit specific ^{125}I - $F(ab')_2$ binding to chick myotube cultures is shown in Table 17. The treatment increased the ability of the receptor to inhibit $F(ab')_2$ binding by 20%. The presence of sodium metaperiodate also slightly increased the specific binding of ^{125}I - $F(ab')_2$ to the cultures, while the oxidation buffer and polyethylene glycol had no effect on $F(ab')_2$ binding, or its inhibition

FIGURE 47. The effect of heat inactivation on the specific binding of Torpedo marmorata AChR to ^{125}I-F(ab')$_2$ fragments.

□ Percentage of ^{125}I-F(ab')$_2$ binding to Torpedo AChR (0.2pmol) remaining after heat inactivation of the receptor.

■ Percentage of ^{125}I-F(ab')$_2$ binding to Torpedo AChR (2pmol) remaining after heat inactivation of the receptor.

▲ Percentage inhibition of specific ^{125}I-F(ab')$_2$ binding to chick embryonic myotube cultures by heat inactivated Torpedo AChR.

--- Percentage of ^{125}I-α- bungarotoxin binding remaining after heat inactivation of Torpedo AChR.

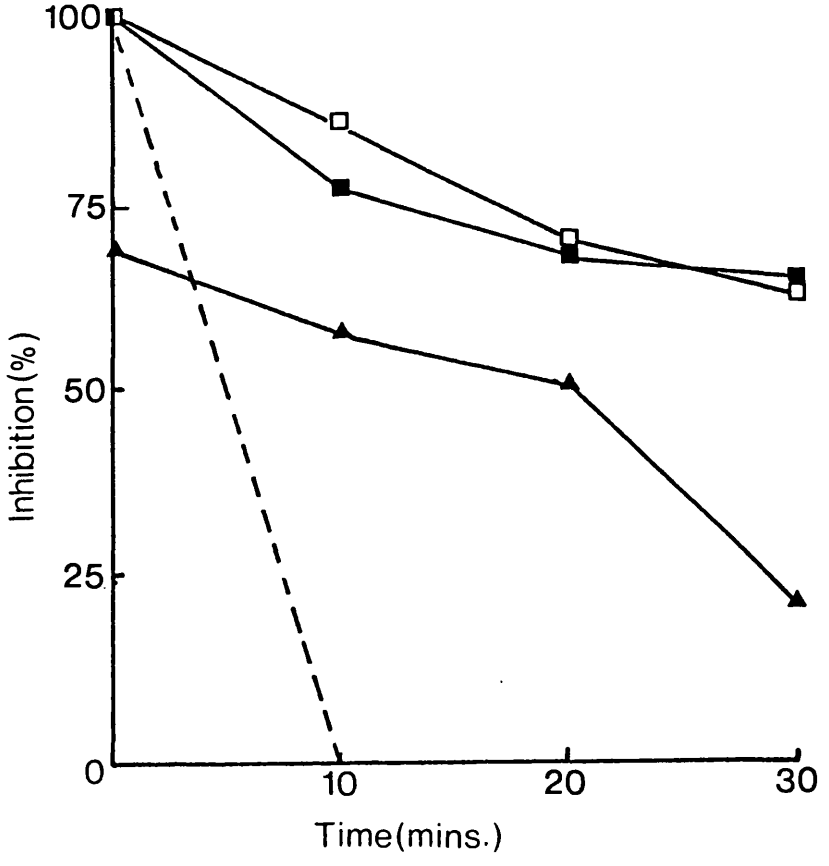


FIGURE 48. Specific binding of SDS-denatured Torpedo marmorata AChR to anti-AChR ^{125}I-F(ab')$_2$.

■ Native AChR

□ SDS-denatured AChR

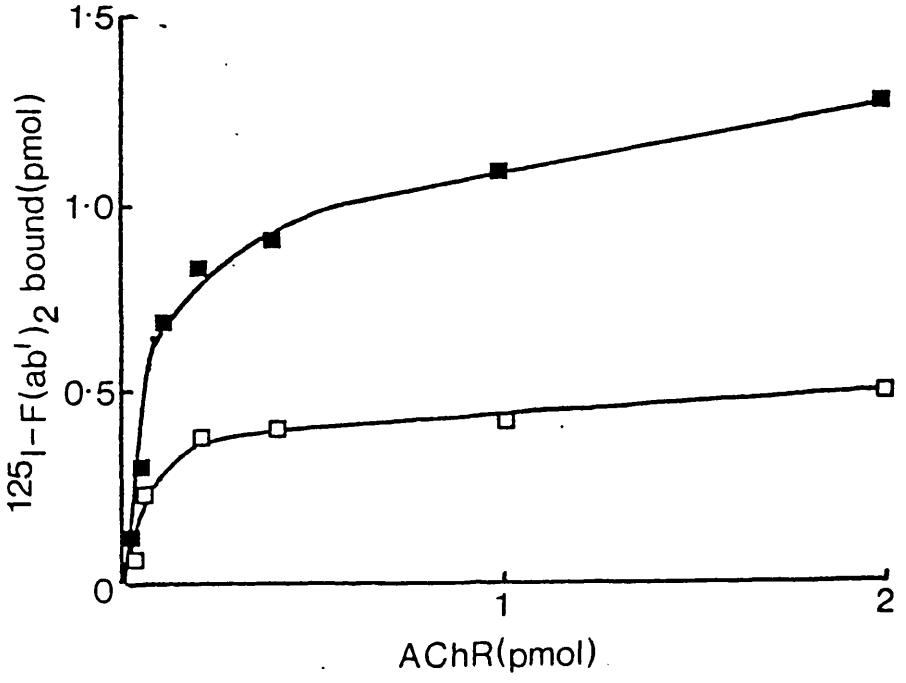


FIGURE 49. The effect of periodate-oxidation of Torpedo marmorata AChR on its specific binding to anti-AChR ^{125}I-F(ab')$_2$.

■ Untreated AChR

□ AChR treated with sodium metaperiodate

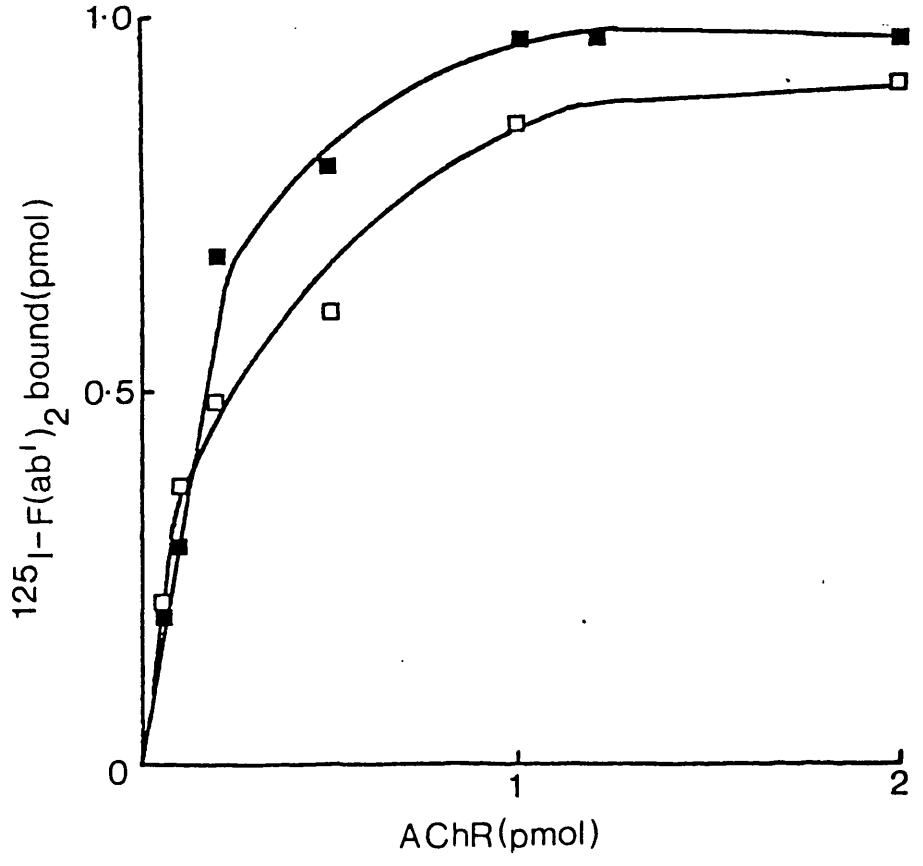


TABLE 17. Effect of periodate oxidation on the ability of Torpedo AChR to inhibit the binding of ^{125}I-F(ab')$_2$ to chick embryonic myotubes.

Incubation	Inhibition of specific ^{125}I-F(ab')$_2$ binding to myotubes (%)
Unmodified Torpedo AChR	70 ± 3
Torpedo AChR + periodate (molar ratio = 1:500)	90 ± 1
Torpedo AChR + buffer alone*	73 ± 2
^{125}I-F(ab')$_2$ + buffer alone	0 ± 1
^{125}I-F(ab')$_2$ + periodate (molar ratio = 1:500)	-13 ± 3

*Controls indicating buffer alone included the addition of ethylene glycol to the reaction mixture.

by Torpedo AChR.

Effect of glycosidase treatment on AChR binding to ^{125}I - $F(ab')_2$

Analysis of T. foetus - treated Torpedo AChR by gas liquid chromatography indicated a reduction in the carbohydrate content of the receptor of at least 50% (A. Clements, unpublished results). The binding of concanavalin A to the T. foetus - treated AChR was reduced by 70% (Wonnacott et al., 1980a).

Figure 50 shows that T. foetus treatment had little effect on the saturation binding of ^{125}I - $F(ab')_2$ to purified Torpedo AChR. The binding assay was performed at 4°C to reduce the effect that any residual glycosidase activity in the treated receptor preparation might have had on $F(ab')_2$ binding to the AChR.

Table 18 shows that T. foetus - treatment of Torpedo AChR did not impair its ability to inhibit specific ^{125}I - $F(ab')_2$ binding to chick myotubes. The binding assay was performed totally at 4°C to reduce the effect that residual glycosidase activity might have had on the assay. Table 18 shows that the presence of T. foetus extract at this temperature had no significant effect on $F(ab')_2$ binding to the myotube cultures.

^3H - Carnitine uptake by chick myotubes in culture

In an initial experiment 10 replicate 7-day cultures of chick myotubes had taken up and retained 4.5 ± 0.6% (mean ± sd) of the initial ^3H - carnitine (^3H-C) added (approximately 30,000 cpm) after the 18h incubation employed in labelling myotubes with ^3H-C. Of this, 39 ± 5% of the retained counts were lost during the further 5h incubation employed in the myolysis assay. The mean retention of ^3H-C in all control cultures at the end of the myolysis assay was 16900 cpm (n = 21 assays of triplicate cultures).

by Torpedo AChR.

Effect of glycosidase treatment on AChR binding to ^{125}I - $F(ab')_2$

Analysis of T. foetus - treated Torpedo AChR by gas liquid chromatography indicated a reduction in the carbohydrate content of the receptor of at least 50% (A. Clements, unpublished results). The binding of concanavalin A to the T. foetus - treated AChR was reduced by 70% (Wonnacott et al., 1980a).

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FIGURE 50. Specific binding of deglycosylated Torpedo marmorata AChR to anti-Torpedo AChR ^{125}I-F(ab')$_2$.

■ Untreated AChR

□ AChR treated with exoglycosidases from Trichomonas foetus.

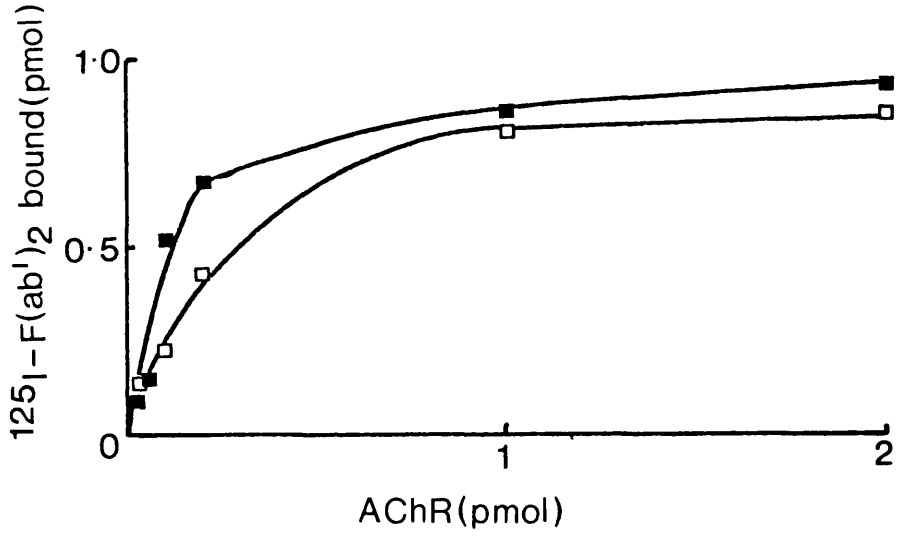


TABLE 18. Effect of glycosidase treatment on the ability of Torpedo AChR to inhibit the binding of ^{125}I-F(ab')$_2$ binding to chick embryonic myotubes.

Incubation	Inhibition of specific ^{125}I-F(ab')$_2$ binding to myotubes (%)
Unmodified *Torpedo* AChR at 4°C	56 ± 3
Unmodified *Torpedo* AChR at 37°C	67 ± 2
Torpedo AChR + *T. foetus* extract at 4°C	57 ± 1
Torpedo AChR + *T. foetus* extract at 37°C	62 ± 5
^{125}I-F(ab')$_2$ + *T. foetus* extract alone	2 ± 8

Lysis of chick myotubes by normal and myasthenic sera

Both normal control and myasthenic sera were highly lytic toward chick myotube cultures, as determined by the loss of ^{3}H-C. The mean lysis caused by 5 normal human sera (5% v/v) not pre-absorbed with chicken liver homogenate was 58% (range = 43 - 79%). Ten myasthenic sera showed a much wider range of myolysis (range = 0 - 70%) with a mean of 35% lysis.

Absorption of sera with chicken liver homogenate

In an attempt to reduce the lytic effects of normal human sera on chick myotubes, sera (1-5ml) were absorbed against a chicken liver homogenate prior to use in the myolysis assay. The myolysis of 5 normal sera (5% v/v) before and after absorption is compared in Table 19. There was a significant reduction in the lytic effects of absorbed sera for 4 out of the 5 sera tested. The lysis of absorbed normal sera at 2.5% v/v was always less than 10%, and this concentration was therefore chosen for comparing the lytic effects of absorbed normal human and myasthenic sera.

Lysis of chick myotube cultures by absorbed sera

To compare the lysis of normal and myasthenic sera after absorption with chicken liver homogenate, 10 normal and 14 myasthenic sera were tested at 2.5% (v/v). The results of this study are shown in Table 20. Normal sera gave a range of mean lysis from 0-6%, with an overall mean for the control group of 3%. For the 14 myasthenic sera the range was greater (0-20%), with an overall mean of 8%. The variation in lysis from assay to assay using the same serum is indicated by the standard deviation of this mean lysis, and was greater for normal sera than for myasthenic sera (average coefficient of variation (cov) 87% and 45% of the mean respectively). The variability in ^{3}H-C retention within triplicates in the same assay, was also higher for normal sera than for myasthenic sera (average cov 75% and 45% of the mean lysis respectively).
There was a statistically significant difference between the lytic activity of control normal sera and that of myasthenic sera ($p<0.05$, student's test). Six of the 14 myasthenic sera gave lytic activity clearly outisde of the normal range, suggesting that focal lysis of the muscle cell membrane may be a significant contributor in the pathology of myasthesia gravis. For the myasthenic sera tested there was no correlation between the anti-AChR antibody titre and lytic activity (Table 20; correlation coefficient $r = 0.04$).

TABLE 19. Lysis of chick embryonic myotubes by normal human sera before and after absorption against chicken liver homogenate.

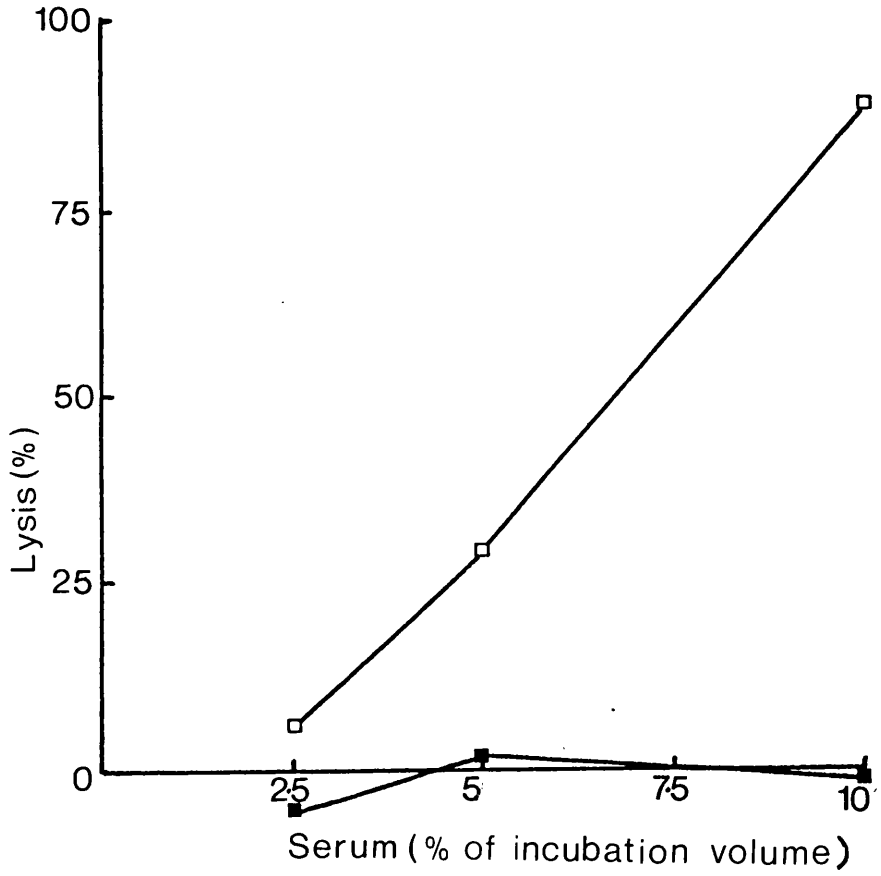
Serum (5% v/v)	Lysis (%)		Reduction in myolysis (%)
	Unabsorbed	Absorbed	
1	43	67	-56
2	50	30	40
3	60	55	8
4	67	37	45
5	79	51	35
mean	60	48	14

Lysis was determined by the retention of ^{3}-carnitine relative to control cultures without serum, and was calculated according to the formula shown in the Methods (p 95).

FIGURE 51. Lysis of myotubes by normal human sera.

□ Lysis of chick embryonic myotubes by serum absorbed against chicken liver homogenate.

■ Lysis of human foetal myotubes by unabsorbed serum.



Lysis was determined by the retention of ^{3}-carnitine relative to control cultures without serum, and was calculated according to the formula shown in the Methods (p 95).

TABLE 20. Lysis of chick embryo myotubes by normal and myasthenic sera absorbed against chicken liver homogenate.

Patient number	Diagnosis	Anti-AChR antibody titre ($x10^{-10}$M)	Lysis (%) mean ± s.d.
1	Normal control	n.d.	0 ± 4 (6)*
2	" "	"	0 ± 2 (6)
3	" "	"	1 ± 2 (12)
4	" "	"	2 ± 3 (6)
5	" "	"	3 ± 4 (6)
6	" "	"	4 ± 3 (5)
7	" "	"	5 ± 2 (6)
8	" "	"	6 ± 2 (6)
9	" "	"	6 ± 2 (6)
10	" "	"	6 ± 3 (11)
11	Generalised M.G.	76	0 ± 3 (5)
12	" "	186	0 ± 1 (2)
13	" "	394	0 ± 2 (7)
14	Ocular M.G.	28	2 ± 3 (6)
15	Generalised M.G.	4	2 ± 1 (2)
16	" "	101	4 ± 3 (5)
17	Ocular M.G.	11	4 ± 3 (3)
18	" "	2	7 ± 4 (6)
19	Generalised M.G.	0	10 ± 2 (6)
20	" "	956	12 ± 2 (6)
21	" "	34	13 ± 3 (6)
22	" "	31	16 ± 4 (6)
23	" "	0	18 ± 2 (6)
24	" "	6	20 ± 2 (5)

n.d. ; not determined

M.G. ; myasthenia gravis

* Figures in brackets indicate the number of determinations

Lysis was determined by incubation of 6-8 day myotube cultures with 2.5% (v/v) serum in the ^{3}H-carnitine retention assay as detailed in the Methods (p 94). Lysis was calculated according to the formula shown in the Methods (p 95).

Effects of heat inactivation and complement activity on serum-mediated lysis of chick myotube cultures

A single myasthenic serum (patient 22, Table 20) was heat-inactivated by incubation at 56°C for 30 min. To test the effects of the heat-treatment on the lytic activity of the serum, a sample of the heat-inactivated serum was compared in the myolysis assay (final concentration 2.5% v/v) with a sample of the same serum taken prior to heat-treatment. The untreated serum had a lytic activity of 15%, while the treated serum gave no lysis, with ^{3}H-C retention equivalent to that of control cultures without serum. Co-incubation of guinea pig complement (Miles) at a final concentration of 10% v/v with the heat-inactivated serum, in the myolysis assay restored the myolysis of the serum to 5% (30% restoration of the original activity of the untreated serum). Since normal human serum was not similarly treated, it is not possible to say whether this restoration of lysis was significant, or was due to the inherent variability of ^{3}H-C retention in the myolysis assay.

Serum-mediated lysis of human foetal myotube cultures

In a preliminary experiment the lytic activity of one normal human serum toward chick embryo myotubes and human foetal myotubes was compared at concentrations of 2.5 - 10% v/v in the myolysis assay (Figure 51). In contrast to the concentration dependent lysis of chick myotubes, even after absorption of the serum against chicken liver, the unabsorbed serum was totally devoid of lytic activity towards human foetal muscle cultures.

The myolysis of human foetal muscle cultures by 3 normal and 6 myasthenic sera (10% v/v) is shown in Table 21. Three of the 6 myasthenic sera showed myotoxicity greater than the maximum lysis obtained with normal sera. The mean lysis of the normal serum (patients 1-3) and myasthenic serum (patients 4-9) groups was 3% and 8% respectively. Due to the small number of samples tested the difference in lysis between the normal serum group and the myasthenic serum group was not statistically significant (Two-sample ranks test, $p > 0.1$). The coefficient of variation between triplicate cultures within the same assay was similar for both normal and myasthenic sera, and averaged 30% over all the samples tested.

TABLE 21. Lysis of human foetal myotubes by normal and myasthenic sera.

Patient number	Diagnosis	Anti-AChR antibody titre ($x10^{-10}M$)	*Lysis (%)
1	Normal control	n.d.	0
2	" "	n.d.	4
3	" "	n.d.	5
4	Myasthenia gravis	n.d.	0
5	" "	n.d.	0
6	" "	18	3
7	" "	571	9
8	" "	186	13
9	" "	2.5	22

n.d. ; not determined.

* Each value represents the mean lysis from a single determination on triplicate cultures of myotubes at 14-21 days in vitro.
Sera were tested for lytic activity at 10% v/v final concentration in the ^{3}H-carnitine retention assay. Lysis was calculated according to the formula shown in the Methods section (p 95).

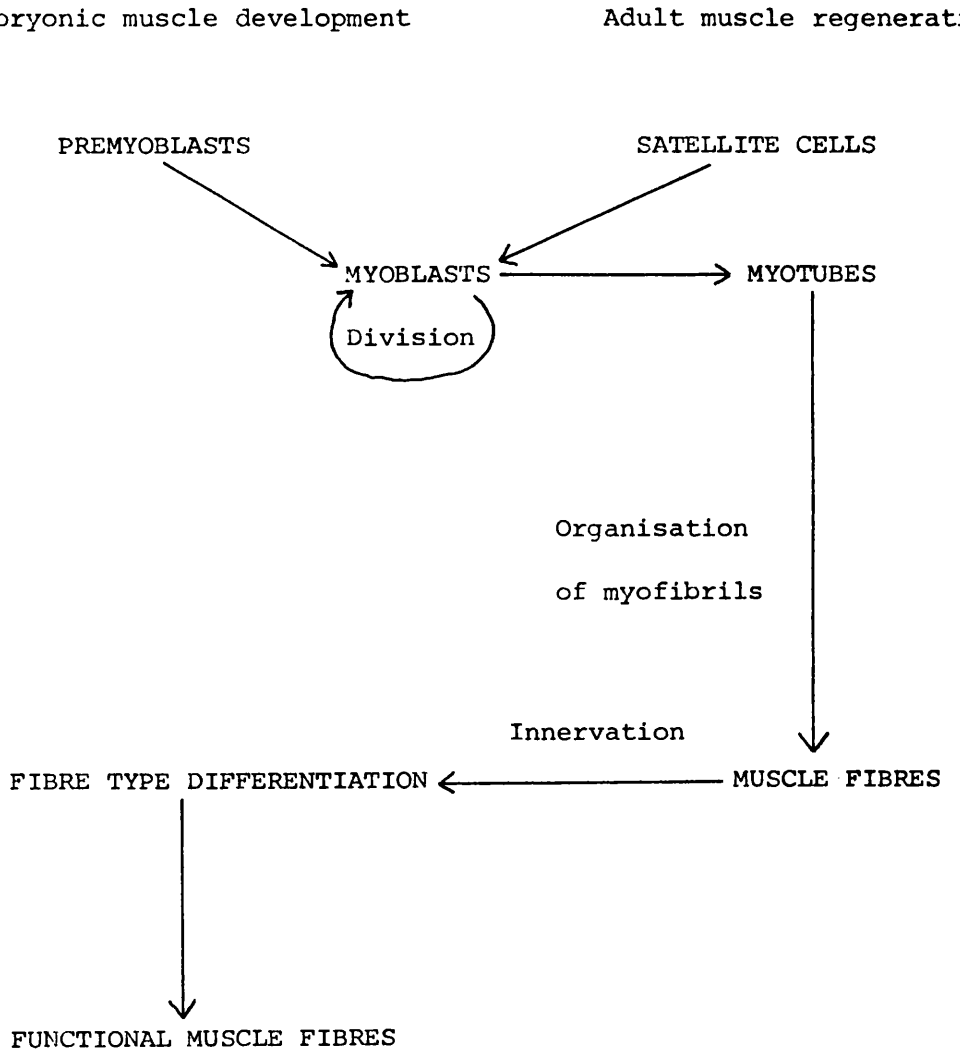
DISCUSSION

Establishment of muscle - cell cultures

The process of myogenesis (Figure 52) has been described in detail. The uninucleate cells that give rise to muscle fibres are myoblasts, and have been prepared from explants of embryonic muscle (Lewis and Lewis, 1917) or by treatment of embryonic muscle with enzymes (Rinaldini, 1959). Myoblasts prepared from adult muscle (Pogogeff and Murray, 1946) are probably derived from satellite cells (Mauro, 1961; Bischoff, 1975). The myoblast cells subsequently fuse to form multinucleated myotubes.

The establishment of myotube cultures from chick embryo skeletal muscle is well documented (for review see Haushka, 1972). When monodispersed muscle cell preparations are used, collagen coating of culture dishes has been found to be essential for good differentiation in culture (Haushka and Konigsberg, 1966; O'Neill and Stockdale, 1972). It seems likely that collagen does not provide any vital nutrients, but rather acts as a superior growth surface for the attachment and growth of cells (Cleator and Beswick, 1972). The growth medium commonly employed is a chemically "undefined" medium which has been found to give optimum growth and differentiation in culture (Yasuko et al., 1981). The presence of embryo extract is essential for myoblast fusion and myotube formation (De La Haba and Amundsen, 1972). A trophic protein (termed "sciatin") isolated from chicken sciatic nerve (Markelonis et al., 1980) was demonstrated to be the major component in chick embryo extract required for myogenesis (Oh and Markelonis, 1980). This trophic factor has now been shown to be identical to the serum protein, transferrin (Markelonis and Oh, 1983).

FIGURE 52. A schematic representation of the process of myogenesis. (After Witkowski,1977).



Myoblast fusion in the cultures used in this study occurred approximately synchronously at about 50 h *in vitro*. This type of rapid burst of fusion has been previously noted by other authors (Morris and Cole, 1972; O'Neill and Stockdale, 1972). At fusion myogenic cells withdraw from the mitotic cycle, with cells due to enter S - phase remaining in G 1 instead, prior to undergoing fusion. Nuclei in the myotubes formed do not synthesise DNA (Morris and Cole, 1972). Thus the rapidly - dividing fibroblasts present in cultures can be killed by a cell cycle phase - specific DNA - synthesis inhibitor, such as cytosine arabinoside (Fischbach, 1972), with no apparent deleterious effect on myotube morphogenesis (Moss *et al.*, 1978). Such treatment of the cultures in this study resulted in a relatively pure myotube population in culture.

Over the past 40 years a number of attempts have been made to grow human muscle cells in culture. Many of these have used adult human muscle, from amputation or biopsy, as a tissue source, and have mostly involved placing explants of chopped muscle into an environment containing growth media and growth substrates (for review see Witkowski, 1977). Cultures derived from single muscle cells or muscle cell pieces obtained by enzymic dissociation of adult muscle have also been established (Morgan and Cohen, 1974; Yasin *et al.*, 1977).

There have been few published reports of cultures derived from human foetal muscle (Kakulas *et al.*, 1968; Haushka, 1974a,b; Emery and McGregor, 1977; Bevan *et al.*, 1977; Cambridge and Stern, 1981). Haushka (1974a,b) used cloning techniques to determine the proportion of myogenic cells present in foetuses of different ages. Muscle cells were prepared by enzymic dissociation and plated at cloning densities.

The percentage of clones containing myotubes and the plating efficiency both rose with increasing foetal age. Minguetti and Mair (1981) showed that human skeletal muscle development *in vivo* falls into two phases. In the first phase from about 9 - 18 weeks the developing muscle consisted mainly of myoblasts and myotubes. In the second phase from 18 weeks onward the muscle was almost entirely fully developed muscle fibres.

In the present study myotube cultures were established from foetal muscle from 8 - 15 weeks. As found by Haushka there was an increase in plating efficiency with increasing foetal age (Tables 8 and 9). Conversely, the cell yield was reduced from limbs of older foetuses.

Methods used to prepare cells for culture depend principally on the age of the donor. In general, explants are preferred for adult tissue, while enzymatic or mechanical dissociation have been used with embryonic or neonatal tissue (Witkowski, 1977). The method of mechanical dissociation used in this study was based on that of Tepperman *et al.* (1975) for chick embryonic muscle. These authors found that trypsinisation yielded almost double the number of cells given by mechanical dissociation, and gave better cell survival. This finding is in broad qualitative agreement with the results of this study. The much greater reduction in cell yield by mechanical dissociation of human foetal muscle in this study reflects the generally increased difficulty in disaggregating human tissue. The relatively long period of trypsinisation necessary to achieve good dissociation is a further confirmation of this fact. Haushka (1972) reviewed enzymic dissociation of normal embryonic skeletal muscle and showed that only trypsin and collagenase had been successfully used. In this study typsinisation resulted in an overall greater cell yield and plating efficiency

than treatment of the tissue with collagenase.

Although media for the growth of embryonic animal muscle are well established, a great variety have been used for human muscle cells (Witkowski et al., 1976). Human muscle cell cultures have been grown in medium 199, Eagle's minimum essential medium, Dulbecco's minimum essential medium, Ham's F10 and Puck's NCI. Clearly, minor variations in composition of the defined medium do not significantly affect muscle cell growth and differentiation (for review see Witkowski, 1977), and this finding is confirmed by the present study. Human muscle cells do not have an obligatory requirement for chick embryo extract, and a number of studies have used media without this supplement (for review see Witkowski, 1977). In this study chick embryo extract was toxic to human muscle cell cultures, in keeping with the findings of Haushka (1972) and Skrbic et al. (1975). Both horse and foetal calf serum have been successfully used in media to grow human myotubes, although horse serum has been generally preferred (for review see Witkowski, 1977). Fibroblastic growth was greatly reduced in cultures grown in media containing horse serum in this study, although myotubes were generally not as well developed as those in cultures grown in media containing foetal calf serum. Attempts to reduce the fibroblastic contamination of cultures grown in the presence of foetal calf serum by either "selective plating" according to the method of Yaffe (1968), or by use of cytotoxic drugs were unsuccessful, as has been previously found (Robertson, J.G. unpublished observation).

Cell cultures prepared from muscle are a heterogeneous population of cells, and it may be difficult to distinguish myoblasts from fibroblasts (Willmer, 1965). Myogenic cells, however, are characterised by their ability to fuse, and the multinucleate myotube can be

unequivocably recognised as a muscle cell. Myotubes in this study were defined as possessing at least three morphologically normal nuclei, as binucleate cells can arise in cultures of fibroblasts (Witkowski, 1977).

^{125}I - α - BGT as a probe for the AChR

In general, two methods, tritiation and radioiodination, have been used to radiolabel the ligands used in binding studies. Tritium labelling results, in most cases, in a labelled ligand which is biologically indistinguishable from the native ligand. In addition, the long radiochemical half-life of tritium (over 12 years) permits extended storage with no loss of sensitivity due to radioactive decay. However, the specific radioactivities obtained with tritium labelling are much lower than can be achieved by radioiodination. For example, one atom of ^{131}I provides the same number of disintegrations per unit time as 600 atoms of tritium. Of the two isotopes of iodine, ^{131}I and ^{125}I, ^{131}I has a much shorter half-life (8 days compared to 60 days for ^{125}I), and ^{125}I has accordingly been the preferred isotope for radiolabelling. When attempts are made to achieve high specific activities however certain problems can arise which may lead to the labelled ligand exhibiting different behaviour from the parent compound (Hunter, 1978; Schmidt, 1984). For this reason attention has been devoted to characterising the ^{125}I - α - BGT used in this study.

It has been shown that the native α - BGT used for radioiodination was at least 98% pure (Barkas, T. unpublished observation).

The method of iodination used routinely was the chloramine - T method initially devised by Hunter and Greenwood (1962) to produce a radioiodinated product of high specific radioactivity. The ^{125}I -

α-BGT used in this study was labelled to a mean specific radioactivity of 733 Ci/mmol., and had a mean biological activity of 56%. The remaining 44% of radioactivity probably consisted of both ^{125}I non-covalently bound to toxin, and denatured labelled toxin. This fraction was eluted unbound from an ion-exchange column designed to bind α- BGT (Figure 23) and did not bind to Torpedo AChR. The ways in which proteins can be damaged during radioiodination with chloramine - T have been discussed by Hunter (1978). In an attempt to determine the percentage of radioactivity corresponding to non-covalently associated ^{125}I, the labelled α- BGT was precipitated with 6% (w/v) trichloroacetic acid. However, the radioactivity precipitated from N - (propionyl - ^{3}H) propionylated α-BGT, which is 98% biologically active, using this method never exceeded more than approximately 70% (Stephenson, F.A. unpublished observation), and consequently this parameter had limited use in the assessment of non-covalently bound ^{125}I in the ^{125}I - α-BGT preparation.

AChR concentrations determined with the high specific activity ^{125}I - α-BGT used routinely in this study may have been underestimated due to the reduced biological activity of the labelled toxin. Thus, correction of Torpedo AChR concentrations for 100% biological activity resulted in values in good agreement with those obtained using the 98% biologically active N - (propionyl - ^{3}H) propionylated α- BGT (Stephenson, F.A. unpublished observation).

The ^{125}I - α-BGT preparation consisted of a mixture of mono - and di - iodinated species, with mono-iodo α-BGT ($^{125}I_1$ - α-BGT) predominating, together with a small fraction which was presumed to be aggregated ^{125}I - α -BGT (Figure 23a). Chloramine - T iodination has been previously reported to cause aggregation of proteins (Krohn

et al., 1972; Sherman et al., 1974) including α-BGT (Jailkhani et al., 1984). Further evidence for the presence of aggregated toxin is the increased size of the peak attributed to it at a higher Chloramine - T concentration (Figure 23b). $^{125}I_1$ - α—BGT and diiodo - α-BGT ($^{125}I_2$ - α-BGT) have been reported to differ in both the kinetics and affinity of their binding to soluble and membrane-bound AChR (Vogel et al., 1972; Lukasiewicz et al., 1978; Blanchard et al., 1979; James et al., 1980). For this reason, an attempt was made to prepare $^{125}I_1$ - and $^{125}I_2$ - α-BGT, and to compare their binding to AChR. $^{125}I_1$ - α-BGT was found to partially co-chromatograph with unlabelled α-BGT (see Results), as has been previously reported (Blanchard et al., 1979; James et al., 1980). In this study there was no significant difference in the binding at saturation, or of the concentration of toxin necessary to achieve saturable binding, to chick muscle AChR, between $^{125}I_2$ - α-BGT and the unfractionated ^{125}I - toxin preparation, although the rate of binding was not determined. $^{125}I_1$ - α-BGT has been shown to bind to Torpedo AChR with the same rate constant as native toxin (James et al., 1980) indicating that monosubstitution with ^{125}I does not alter the binding properties of α- BGT.

Binding of ^{125}I - α-BGT to myotube cultures

When muscle cultures from chick embryo are grown, α- BGT binding activity remains at a very low level until some 55 - 60% of myoblasts have undergone fusion. AChR elaboration does not, however, depend on fusion per se, since postmitotic, fusion-arrested myoblasts also increase in α-BGT binding activity (Patersen and Prives, 1973). Sytkowski et al. (1974) found that, in normal muscle cultures, toxin binding reached a plateau at 6 - 7 days after plating, and remained constant at this level for at least 10 days in culture. These authors

also found that, at day 5 in culture, AChR's were evenly distributed at a low density over the whole myotube surface. By day 7 discrete clusters of AChR were present on 1% of the myotubes, and by day 11 more than 80% of the myotubes had one or more receptor clusters. On the basis of these findings, chick myotube cultures of 6 - 8 days *in vitro* were used in all the experiments reported in this thesis, in an attempt to have a relatively homogeneous population of AChR for study.

Chick myotubes in culture respond to agonists that normally activate nicotinic cholinergic receptors at the neuromuscular junction, but not to agonists that do not normally act on skeletal muscle. All of the nicotinic AChR stimulant drugs tested to date have produced a response. This includes decamethonium, succinylcholine and pilocarpine (Harvey and Dryden, 1974). The response of cultured muscle to acetylcholine is blocked by the nicotinic antagonist, d - tubocurarine, at low concentrations, but by atropine only at concentrations of 100 - 1000 times the effective tubocurarine concentration (Dryden,1970). Elson (1979) has noted that the pharmacological properties of early (10 day) embryonic chick AChR have not been fully characterised.

In initial work to determine ^{125}I - α-BGT binding to chick myotubes in this study, non-specific binding was measured in the presence of d - tubocurarine (10^{-4} M final concentration). The maximum protection of toxin binding obtained was 76%. Decamethonium bromide (10^{-5} M final concentration) inhibited ^{125}I - α-BGT binding by an average of 87%, and was consequently used in preference to tubocurarine. The greater protection afforded by decamethonium over tubocurarine has been previously reported in this system (Vogel *et al.*, 1972; Paterson and Prives, 1973).

After binding of ^{125}I -α-BGT to myotube cultures, the labelled cell monolayer has been suspended by mechanical methods (Patrick et al., 1972), or by solubilisation with trypsin (Vogel et al., 1972; Paterson and Prives, 1973) or NaOH (Elson, 1979). In this study the most consistent results were obtained with NaOH suspension of the cells. A 60 min incubation with ^{125}I -α-BGT was performed to allow maximum binding of the toxin. A number of reports have shown that maximum binding is obtained in about 20 - 30 min (Vogel et al., 1972; Patrick et al., 1972; Paterson and Prives, 1973), and a 60 min incubation time has been routinely used (Vogel et al., 1972; Sytkowski et al., 1974).

The maximum specific binding of ^{125}I - α-BGT in this report was relatively consistent from culture to culture (0.36 $\pm$ 0.06 pmol/mg cell protein; n = 10). The binding curve obtained was very similar to that of Vogel et al. (1972) who used chick embryo muscle cultures at 12 days in vitro. The K_D of ^{125}I - α-BGT binding to chick myotubes was calculated to be 2.5 x 10^{-9} M. This is of the same order as that calculated from the study of Vogel et al. (1972) (1 - 2 x 10^{-9} M) and the K_D of high affinity sites (7.2 x 10^{-9} M) determined by Elson (1979).

Human myotube cultures have been shown to respond to iontophoretic application of acetylcholine, but not to the muscarine stimulant, acetyl - β - methylcholine (Robertson, J.G. unpublished observation). However, few reports of α- BGT binding to human muscle in culture have appeared. In this study the binding of ^{125}I -α-BGT to human myotubes was much less, and of reduced affinity, compared to chick myotubes (Figures 25 and 48). In addition, the protection offered by decamethonium was also considerably lower than in the case

of chick embryo muscle cultures (a 100 - fold excess concentration was necessary to block ^{125}I - α - BGT binding significantly). Previous studies of human muscle in tissue culture have not obtained very mature fibres. For example, the frequency of occurrence of cross-striations and spontaneous contractions, where reported, has not been high (Witkowski et al., 1976; Crain et al., 1970; Yasin et al., 1977). Cross-striations only appeared in adult human myotubes after a long period in culture (Pogogeff and Murray, 1946). In the dissociated cell cultures reported here, maintenance for more than 2 - 3 weeks was difficult because of three major problems: (1) Fibroblastic over-growth of cultures, (2) Fungal contamination and (3) Autolysis of myotubes, so that the myotubes did not have sufficient time for extensive maturation. The low level of α- BGT binding to the cultures is therefore probably a reflection of the limited development of the myotubes in culture.

F(ab')$_2$ and Fab binding to AChR

F(ab')$_2$ and Fab fragments of IgG were prepared from the sera of both normal sheep (non-immune fragments), and of sheep immunised with Torpedo AChR and exhibiting symptoms of EAMG (immune fragments). Both immune and non-immune F(ab')$_2$ and Fab chromatographed as single molecular weight species in SDS - polyacrylamide gel electrophoresis, indicating their purity through the separation procedure. The immune F(ab')$_2$ fragments retained the ability of the original antiserum to recognise and bind AChR. This was confirmed by their total inhibition of precipitation of ^{125}I - labelled Torpedo AChR by rabbit antiserum to Torpedo receptor, while non-immune F(ab')$_2$ fragments were without effect (Wonnacott et al., 1980a).

Both intact IgG and F(ab')$_2$ fragments from anti-AChR antisera

have been shown to cause increased degradation of AChR from the membranes of cultured muscle cells (Heinemann et al., 1977; Prives et al., 1979; Patrick and Berman, 1980). This phenomenon is known as "antigenic modulation". Immune $F(ab')_2$ fragments caused antigenic modulation of chick myotube cultures at 37°C (Figure 36), which was very similar to that described by Prives et al. (1979) for rabbit anti-AChR $F(ab')_2$ fragments. This further confirmed that the $F(ab')_2$ fragments were not functionally impaired by the purification procedure.

To study the direct binding of $F(ab')_2$ and Fab fragments to unmodified AChR, the fragments were labelled with ^{125}I by the method of McConahey and Dixon (1966), which was designed to cause minimum functional damage to the protein labelled. Early attempts to iodinate $F(ab')_2$ fragments were performed in Tris/HCl buffer, pH 8.0, and resulted in a poor incorporation of ^{125}I into the protein. This was probably because the pH in the iodination reaction rose above 8, where the efficiency of labelling falls off rapidly (Hunter, 1978). Reduction of the pH by use of a phosphate buffer resulted in a much greater incorporation of ^{125}I into the $F(ab')_2$.

Immune ^{125}I - $F(ab')_2$ fragments bound saturably to purified Torpedo AChR (Figure 37b) while non-immune ^{125}I - $F(ab')_2$ showed little binding above that of controls from which AChR was excluded. Excess of Torpedo AChR bound approximately 5% of the total immune ^{125}I - $F(ab')_2$ added, corresponding to the fraction of the $F(ab')_2$ preparation representing specific anti-AChR antibody fragments, as determined by affinity purification. This indicated no impairment of function of the $F(ab')_2$ fragments after labelling with ^{125}I. The specificity of immune ^{125}I - $F(ab')_2$ binding to the

receptor was confirmed by the almost complete inhibition of the binding given by unlabelled immune or affinity - purified $F(ab')_2$ fragments. In contrast non-immune $F(ab')_2$ or the unbound fraction from the affinity column were without effect. The affinity purified $F(ab')_2$ fragments maximally inhibited the binding of ^{125}I - $F(ab')_2$ fragments to AChR at considerably lower protein concentrations than required for the parent fragments to achieve the same effect, and represented an approximately 6 - fold purification of the immunospecific $F(ab')_2$ fragments. It is not clear why a higher purification was not obtained. One possibility is that some inactivation occurred in the presence of 0.2 M NH_4OH during elution from the affinity column. A Scatchard plot of ^{125}I - $F(ab')_2$ binding to purified Torpedo AChR

gave an average intrinsic dissociation constant of 2.5×10^{-8} M, which is of the same order as the K_D's reported by Lindstrom et al. (1981) for mAb binding to isolated Torpedo AChR.

In the present study immune ^{125}I - $F(ab')_2$ and Fab bound specifically to chick myotubes in culture. Non-specific, or non-receptor binding to the myotubes was determined from the binding of non-immune ^{125}I - fragments. This was the only practical measure of non-specific binding possible, because of the large concentration of ^{125}I - $F(ab')_2$ needed to achieve saturable binding, and the relatively long incubation time employed in the binding assay. Aarli (1970) reported that muscle-binding γ- globulins from normal human sera bound to the tissue only by the Fc portion of the molecule, and this was part of the rationale for using $F(ab')_2$ fragments to determine

binding to the AChR on chick myotubes. Nevertheless the binding of non-immune ^{125}I - $F(ab')_2$ and Fab to the myotubes was considerable, although it was non-saturable.

A cross reactivity between the foreign immunizing AChR (from fish electric organ) and self muscle receptor is required for the induction of the autoimmune process in EAMG. It is thus important to study those antibodies that cross-react with muscle AChR (Souroujon et al., 1983). As expected, the cross reactivity between the anti - (Torpedo AChR) $F(ab')_2$ fragments and chick myotube AChR was quite low. This is shown by the stoichiometry of the reactants (amount of AChR: amount of specific anti-AChR $F(ab')_2$) at saturation of the receptor with ^{125}I - $F(ab')_2$, which was approximately 2:1 for binding to purified Torpedo AChR but 1:100 for binding to myotube AChR, giving a cross reactivity of 0.5%, in keeping with the low levels of cross-reactivity reported previously (Lindstrom et al., 1978, 1979b; Vincent, 1980; Tzartos et al., 1981). This suggests substantial differences in the immunogenic properties of the two receptors. Differences in the antigenic determinants between solubilised and membrane-bound AChR (Martinez-Carrion et al., 1981) may also contribute to the lack of cross-reactivity. A Scatchard plot of specific ^{125}I - $F(ab')_2$ binding to myotube cultures showed a single class of binding sites with a K_D of 1×10^{-7} M. This is again consistent with a limited cross-reactivity between antibody binding to Torpedo and chick muscle AChR, and is in keeping with the findings of Tzartos and Lindstrom (1981) and Tzartos et al. (1981) who found that most of a panel of mAbs to Torpedo AChR which were capable of cross reacting with AChRs from other species were directed at the "main immunogenic region" on the receptor.

In an attempt to develop a binding - inhibition assay which could be used in conjunction with AChR modification to probe the

nature of the cross-reacting antigenic sites on chick myotube AChR, purified Torpedo AChR was preincubated with ^{125}I - $F(ab')_2$ fragments prior to assaying binding of the $F(ab')_2$ to chick myotube cultures. Under these conditions binding was inhibited by a maximum of approximately 80% and indicated clearly that ^{125}I - $F(ab')_2$ binding to the myotubes was specifically directed at the AChR. One possible explanation for the lack of complete inhibition is that a percentage of the $F(ab')_2$ molecules bound to the Torpedo AChR by one arm only, leaving the other arm of the divalent molecule free to interact with the myotube receptor. The 20% remaining $F(ab')_2$ binding to myotube cultures would then be a purely statistical function based on a random binding of $F(ab')_2$ to the available sites on the Torpedo receptor.

^{125}I - Fab fragments bound much more variably and with a lower affinity to chick myotubes than ^{125}I - $F(ab')_2$.

This is not surprising since the binding affinity of Fab fragments to antigen is known to be generally significantly lower than that of the parent immunoglobulin (Eisen, 1974). The variability of binding may indicate a variable degree of damage to the protein during the purification and iodination procedure.

Interrelationship of the antigenic and toxin binding sites of the AChR

The interaction of an enzyme with a specific antibody usually leads to a reduction in enzyme activity (for review see Arnon, 1973).

This has its counterpart for the AChR in the direct blockade of cholinergic ligand binding to the receptor by anti-AChR antibodies. Such a blockade could be an important factor in the pathogenesis of myasthenia gravis, and would explain the curare-like neuromuscular block evident early in the course of the disease, before any structural

damage at the synapse can be demonstrated. Changes in the levels of antibodies specific for the cholinergic binding site may also account for the rapidly occurring clinical exacerbations and remissions seen in myasthenia, and for the immediate improvement that occasionally follows a single plasmapheresis treatment.

Myasthenic sera have been shown to contain antibodies which inhibit α- BGT binding to both solubilised and membrane-bound AChR (for review see Vincent, 1980). The percentage of myasthenic patients possessing such antibodies has been reported to range from 0 (Harvey et al., 1978) to 65% (Bradley et al., 1979). In a study of 12 myasthenic sera, Whiting et al. (1983) found that anti -α-BGT site antibodies varied from 0 - 33% of the total anti-AChR antibody population, while Dwyer et al. (1979) reported 4 patients in which anti-toxin site antibodies represented 51 - 100% of the total population. The presence of antibodies capable of blocking α- BGT binding to the receptor in myasthenic patients has been shown to correlate with disease severity (Lefvert et al., 1981; Drachman et al., 1982). Lefvert et al. (1981) showed that most patients exhibiting anti-toxin site antibody activity had disease in later stages (II B, III or IV) of the Osserman classification. These authors also found that antibodies to the ligand binding site were mostly found in subclasses 1 and 3. More recently, however Whiting et al. (1983) found that such antibodies were restricted to subclass 3 in only 2 of 12 patients.

The ability of anti-AChR antibodies in EAMG to inhibit toxin binding to the receptor has varied from 0 - 100% for membrane bound AChR (Lindstrom, 1977; Zurn and Fulpius, 1977; Karlin et al., 1978; Eldefrawi, 1978) and from 25 - 100% for solubilised receptor (Zurn and Fulpius, 1977; Harvey et al., 1978; Claudio and Raftery, 1980; Penn et al., 1976; Aharonov, 1977; Wonnacott et al., 1980b).

Most studies have shown an incomplete inhibition of toxin binding to the AChR even in extreme antibody excess. This is a characteristic feature of antibody - enzyme interactions (Cinader, 1963). Incomplete inhibition of ^{125}I -α-BGT binding to both purified Torpedo AChR and chick myotube AChR was also found in this study. The incomplete inhibition of toxin binding by anti-receptor antibodies has led some authors to postulate the existence of heterogeneity in the AChR population with respect to antigenic specificity (Mittag et al., 1981a) but may also be explained as a simple statistical function resulting from random binding of a heterogeneous population of anti-AChR antibodies to the receptor. Thus the binding of one antibody molecule might sterically hinder or prevent the binding of a second antibody molecule overlying the toxin binding site (Figure 53). Such a mechanism has also been proposed by James et al. (1983).

There are three mechanisms by which anti-AChR antibodies could inhibit toxin binding to the AChR. Firstly antibody molecules could directly compete with α- BGT for the same site. Secondly, the antibody might bind at a site close to the toxin binding site and sterically hinder the binding of the toxin molecule. Thirdly, antibody might bind at a site remote from the toxin binding site but alter the conformation of the receptor in such a way as to modify its toxin binding capacity.

In this study ^{125}I - α-BGT binding to chick myotube AChR in situ could be inhibited by anti-AChR $F(ab')_2$ fragments but not by Fab fragments. The lack of inhibition of toxin binding to membrane-bound AChR by Fab fragments was also found by Claudio and Raftery (1980) and Martinez-Carrion et al. (1981), but is not due to an inability of Fab to bind to the AChR (Martinez-Carrion et al., 1981 and Figure 33).

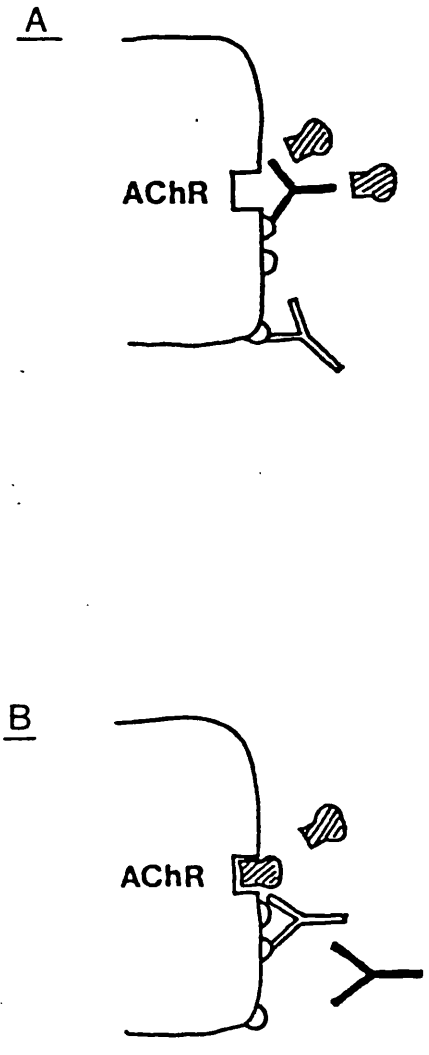
FIGURE 53. A schematic model for the steric inhibition of α- bungarotoxin binding to AChR by anti-receptor antibody.

α- bungarotoxin

anti-"toxin site" antibody

anti-AChR antibody directed at other antigenic sites.

In A the binding of toxin is blocked by the anti-"toxin site" antibody, while in B the anti-site antibody is prevented from binding by a second antibody directed away from the toxin-binding site, thus leaving this site free to bind toxin.



The inhibition of α- BGT binding by $F(ab')_2$ fragments was not due to antigenic modulation of the AChR, since no increase in receptor degradation in the presence of immune $F(ab')_2$ was observed under the conditions of the inhibition assay (Figure 36). This is not surprising since the assay was performed at 4°C ($F(ab')_2$ binding), followed by 25°C (toxin binding) for only a short time, and the process of modulation has a strict temperature dependence (Baumann and Doyle, 1980) and is not significant for AChR at 23°C or below (Heinemann et al., 1977; Appel et al., 1977). The results obtained in this study are consistent with a steric block of α- BGT binding by the anti-AChR antibodies, rather than a direct competition for the same combining site on the receptor. This is further confirmed by the fact that preincubation of chick myotubes with α- BGT had no effect on ^{125}I - $F(ab')_2$ binding. This finding is similar to the small (5 - 15%) inhibition of ^{125}I - labelled anti-AChR antibody binding to Torpedo marmorata membrane fragments by α- BGT reported by Zurn and Fulpius (1977). The fact that $F(ab')_2$ but not Fab fragments inhibited toxin binding in this system indicates that the sterically hindering antigenic site is not in very close proximity to the α- BGT binding site. As the surface diameter of the AChR is about 8.5 nm (Kistler et al., 1982) and the maximum distance between the two arms of an IgG molecule is of the order of 12 nm (Edelman and Gall, 1969) it is theoretically possible for an antibody bound at any given receptor determinant to cover any other region of the receptor surface.

Both immune $F(ab')_2$ and Fab fragments inhibited binding of ^{125}I - α-BGT to purified Torpedo AChR. For Fab fragments this is in agreement with the findings of Claudio and Raftery (1980), who demonstrated a 26 - 70% inhibition of α- BGT binding to Torpedo AChR by Fab fragments from anti - (Torpedo AChR) antisera raised in both

rabbits and rats. In contrast Mihovilovic and Martinez-Carrion (1979) demonstrated no inhibition of toxin binding by high affinity anti-AChR Fab fragments, although the time dependence of toxin binding was changed suggesting some overlap between the Fab and toxin combining sites. As in the case of chick myotubes, preincubation of Torpedo AChR with α- BGT did not inhibit the binding of ^{125}I - $F(ab')_2$ to the receptor. The inhibition of toxin binding in this system, therefore, again appears to be by steric hindrance of the α- BGT binding site by antibody bound at an adjacent antigenic site.

The curves for inhibition of binding of ^{125}I - α- BGT by $F(ab')_2$ and Fab fragments when analysed by a Hill plot all gave Hill coefficients close to unity , indicating one molecule of antibody bound per toxin binding site. Using a similar analysis, Mittag et al. (1981a) found two antibody molecules bound per toxin site in myasthenic sera containing anti-toxin site antibodies.

The conformational state of the AChR is important for the inhibition of toxin binding by anti-receptor antibodies. (Bartfield and Fuchs, 1979; Claudio and Raftery, 1980). In this study the lack of inhibition of toxin binding to chick myotube AChR by Fab fragments, in contrast to the 80% inhibition to purified Torpedo AChR suggests that the membrane-bound and solubilised AChR are antigenically different. Differences in the binding of high affinity anti-AChR Fab fragments and mAb's to solubilised and membrane-bound receptor have been previously reported (Martinez-Carrion et al., 1981; Souroujon et al., 1983), and could result from the exposure of hidden antigenic determinants by the solubilisation procedure.

Interrelationship of carbohydrate and antigenic sites on AChR

Membrane-bound carbohydrate is known to contribute to cell surface specificity in a wide range of systems (Harrison and Lunt, 1980). Impairment of AChR glycosylation in cultured muscle has been shown to hinder accumulation of the receptor in the cell membrane by both preventing the assembly of subunits into a functional macromolecule (Merlie et al., 1982), and increasing the susceptibility of the AChR to degradation by cellular proteases (Prives and Olden, 1980). The interrelationship between carbohydrate and the α- toxin binding site on the AChR has been studied by a number of investigators. Concanavalin A, a ligand specific for carbohydrate moieties, binds to the AChR at multiple, cooperative binding sites (Mittag et al., 1981a) and causes inhibition of α- toxin binding to both solubilised and membrane-bound receptor (Wonnacott et al., 1980b; Mittag et al., 1981; Boulter and Patrick, 1979). In these studies the inhibition was incomplete, ranging from 25 - 90%, and was postulated to be due to steric hindrance rather than direct involvement of carbohydrate residues in the α- toxin recognition site (Wonnacott et al., 1980b). This was confirmed by the finding that the equilibrium and kinetic properties of AChR - toxin complex formation were not modified by treatments which caused the destruction or removal of carbohydrate residues from the receptor (Wonnacott et al., 1980b; Criado and Barrantes, 1982). In the present study, Concanavalin A caused a maximum 60% inhibition of ^{125}I -α-BGT binding to chick myotubes, indicating a similar juxtaposition of carbohydrate to the toxin binding site in this system as in those previously reported.

The contribution of carbohydrate to the antigenicity of the AChR has also been examined. Mittag et al. (1976, 1981b) reported that 65% of myasthenic patients possessed anti-AChR antibodies which

interfered with the binding of labelled AChR to Concanavalin A coupled to beaded agarose gel. Similarly Wonnacott et al. (1980a) showed that the binding of Concanavalin A to ^{125}I - labelled Torpedo AChR was specifically inhibited to a maximum of 25% by anti - (Torpedo AChR) $F(ab')_2$ and Fab fragments. Dwyer et al. (1981a) and Hall et al. (1983) both found that some myasthenic sera inhibited the binding of α- BGT to extrajunctional rat AChR to a greater extent than to junctional receptor, and that this difference was reduced or abolished by glycosidase treatment of the receptor. Taken together, these findings indicate that carbohydrate may be one of many antigenic sites on the AChR. To clarify further the role of carbohydrate in the antigenicity of the AChR, the direct effects of removing carbohydrate groups from the receptor, on ^{125}I - $F(ab')_2$ binding to purified Torpedo AChR, and to chick myotube AChR in situ was studied. Neither periodate oxidation, nor glycosidase treatment of Torpedo AChR reduced its ability to bind directly to labelled anti-AChR $F(ab')_2$ fragments, or to inhibit the binding of the fragments to chick embryo muscle in culture. The efficacy of the treatments was, however, evidenced by a 45 and 70% reduction in binding of the receptor to Concanavalin A by periodate and glycosidase treatments respectively. These findings are in good agreement with those previously reported using AChR labelled with either ^{125}I -α-BGT, or directly with ^{125}I, as antigen (Weinberg and Hall, 1979; Wonnacott et al., 1980a; Dwyer et al., 1981a). These results, therefore, show little evidence of a role for the carbohydrate component of the AChR as a major antigenic determinant, and indicate that the antibodies to Torpedo AChR used in this study are primarily directed at sites other than the carbohydrate itself.

The effect of AChR - denaturation on antigenicity

Atassi (1975, 1978) demonstrated that the antigenic determinants of a protein are generally formed by six or seven amino acid residues. If these residues are adjacent the determinant is termed "continuous". If the amino acids forming the determinant are from different parts of the primary sequence, and the determinant is formed by folding back of the polypeptide chain or by residues from adjacent chains, the determinant is termed "discontinuous". Antibodies directed against continuous determinants would be expected to bind

to the denatured protein, whereas antibodies to discontinuous determinants, which are conformationally dependent, would bind only to the intact, native protein.

To determine whether the immune ^{125}I - $F(ab')_2$ fragments used in this study were directed primarily at continuous or non-continuous determinants, the effect of AChR denaturation on the binding of $F(ab')_2$ fragments to the receptor was studied.

SDS - treatment of Torpedo AChR resulted in a 65% decrease in the maximum amount of ^{125}I - $F(ab')_2$ directly bound to the receptor, while heat-inactivation at 60°C for 30 min caused only a 35% loss of $F(ab')_2$ binding. In contrast, the toxin binding capacity of the receptor was almost completely abolished by a 10 min exposure at this temperature, as has been previously reported (Saitoh et al., 1979). The loss of antigenicity on denaturation is in keeping with the findings of Bartfield and Fuchs (1977), who analysed the antigenic specificities of untreated and reduced, carboxymethylated Torpedo AChR, and found that some antigenic determinants were abolished by the denaturation procedure. These determinants may be functionally important, since denatured AChR does not induce any

symptoms of EAMG in inoculated animals (Valderrama et al., 1976; Lindstrom et al., 1976c; Bartfield and Fuchs, 1977; Claudio and Raftery, 1980).

The ability of Torpedo AChR denatured by SDS - treatment, or exposure at 60°C to inhibit the binding of ^{125}I - $F(ab')_2$ to chick myotubes was decreased to a greater extent than its ability to directly bind the $F(ab')_2$ fragments (to 16 and 30% of the inhibition caused by intact receptor for the two treatments respectively). This accords with Lindstrom et al. (1979b) who found that most interspecies cross-reaction occurred at conformationally dependent determinants, and suggests that the interactions of the anti - (Torpedo AChR) antibody fragments used in this study with the receptor in situ in chick myotubes are primarily directed at conformational sites on the receptor. Such sites are significantly immunogenic and hence may be of particular relevance to the pathogenesis of myasthenia gravis.

Lysis of muscle cells in culture by myasthenic sera

Anti-AChR antibody is present in over 85% of myasthenia gravis patients and is thought to be responsible for the reduction in end-plate AChR which underlies the basic defect in neuromuscular transmission. Anti-receptor antibodies may reduce the number of available receptors by three main mechanisms.

1) By direct blockade of AChR function, resulting from antibody binding to the acetylcholine binding site, the ion-channel or to some important conformational determinant.

2) By increasing the rate of AChR degradation (antigenic modulation).

3) By antibody - mediated damage of the AChR and lysis of the post-synaptic region, possibly in conjunction with complement or cellular elements.

The evidence available suggests that all three mechanisms may be involved in the pathogenesis of myasthenia gravis. The contribution of direct blockade of AChR function, and of antigenic modulation to AChR loss have been extensively documented, but are both insufficient, by themselves, to account for the development of muscle weakness in myasthenia gravis and EAMG (Berman and Heinemann, 1984; Lindstrom, 1979). By contrast the contribution of antibody - mediated lysis of the postsynaptic membrane has been relatively little studied, despite the proposal that this may be the most important mechanism operating in both acute EAMG and myasthenia gravis (Lindstrom and Engel, 1981; Drachman, 1981). For this reason the lysis of muscle cells in culture by myasthenic sera has been studied.

The identification of specific myotube damage in inflammatory muscle diseases and myasthenia gravis has been attempted by direct light microscopic assessment (Currie, 1970; Liveson et al., 1976) or by studies of chromium - 51 release from labelled muscle cultures (Dawkins and Mastaglia, 1973). However, damage to fibroblasts, which generally contaminate myotube cultures, cannot be distinguished from myotube damage by these methods, leading to inaccuracies in the determination of muscle - specific lysis. An alternative approach would be to employ a compound selectively taken up by myotubes and likely to be released rapidly following myotube damage. Carnitine (γ- amino - β - hydroxy butyric acid 3 - methyl - betaine), though found in a number of cell types, occurs in particularly high concentration in muscle cells (Greville and Tubbs, 1968). Both cardiac and skeletal muscle cells take up carnitine by means of a membrane - associated active transport system (Rebouche, 1977). The uptake of tritiated (^{3}H) - carnitine into human foetal muscle cells in culture has been studied (Cambridge and Stern, 1981). Uptake into myotubes

was approximately 5 - times higher than that into fibroblast cultures, and was much more efficient, with a 7 - fold higher Km/Vmax ratio. Specific ^{3}H - carnitine loss from myotubes following incubation with lymphocytes from patients with inflammatory muscle disease was also demonstrated by these authors. The method was considerably more sensitive than either visual assessment of cell lysis, or loss of chromium - 51,since only myotubes were labelled to any significant extent, and loss of membrane integrity not gross enough to be microscopically visible could be detected. The loss of ^{3}H - carnitine from myotube cultures was accordingly used to determine antibody - mediated myotoxicity in this study.

Both normal human and myasthenic sera caused myolysis of chick embryo muscle cultures. Lysis of chick myotubes by normal sera was also observed by Harvey et al. (1978), and suggests the widespread occurrence of natural anti-chick antibodies. Absorption of the normal sera with chicken liver homogenate considerably reduced their myolytic activity, in keeping with the findings of Harvey et al. (1978).

In a study of the lytic effects of myasthenic sera on mouse somite cultures containing mature cross-striated muscle fibres Liveson et al. (1976) found 3 of 17 sera which caused partial destruction of the cross-striated fibres observable by light microscopy. Cytolysis of the muscle cells in excised frog nerve-muscle preparations by 2 of 22 myasthenic sera was also observed by Nastuk et al. (1959). In the present study, 6 of 14 myasthenic sera previously absorbed against ckicken liver possessed lytic activity which was clearly outside of the normal range. The greater percentage of positive sera than shown in the two previous reports cited is prob-

ably a reflection of the increased sensitivity of the ^{3}H - carnitine release assay as a measure of cytotoxicity in muscle cultures. The myolysis observed was not related to anti-AChR antibody titre of the myasthenic serum, with two of the lytic sera having no detectable titre. This suggests that myolysis may be caused by antibodies to muscle components other than the AChR, which are present in up to 70% of myasthenic sera (Mehl and Lang, 1984). An alternative possibility is that lysis is mediated by antibodies directed at the toxin binding site of the AChR. These antibodies are not detected by the immunoprecipitation assay commonly used to determine anti-AChR antibody titre in myasthenic sera, as the antigen used is a receptor-toxin complex.

Complement may play a role in antibody - mediated damage to muscle cells. Research on complement - deficient mice, rabbits and guinea pigs indicates that complement plays an important role in EAMG. Acute EAMG is prevented in rats depleted of C3 by treatment with cobra venom factor (Lennon et al., 1978). Similarly guinea pigs that were C4 - deficient developed normal titres of antibody against Torpedo AChR, but did not exhibit clinical symptoms (Eldefrawi, 1978). In myasthenia gravis focal lysis of the postsynaptic membrane can be observed ultrastructurally, with fragments containing AChR, antibody and C3 being shed from the membrane into the synaptic cleft (Engel et al., 1977). In this study heat-inactivation of myasthenic sera totally abolished their myolytic activity, and this was only partially restored by the addition of guinea pig complement. Liveson et al. (1976) also found that the addition of complement did not restore the myolytic activity of heat-inactivated sera. In contrast to this Harvey et al. (1978) found that fresh-diluted guinea-pig serum restored the lytic activity of sera heat-inactivated at 56^{o}C for

60 min, and recent results indicate that high levels of complement will restore the myolytic activity of heat-inactivated myasthenic sera against rat myotubes (Childs, L., Harrison, R. and Lunt, G.G. (1985) J. Neuroimmunol. in the press).

In view of the relatively limited cross-reactivity between myasthenic anti-AChR antibodies and chick myotube cultures (Fulpius et al., 1980), the high percentage of myasthenic sera giving lysis suggests that antibody - mediated myolysis may be an important mechanism in the pathogenesis of myasthenia gravis. To further explore the significance of this mechanism, and to try to eliminate the problems of normal serum toxicity in the myolysis assay, an attempt was made to develop human foetal myotubes in culture, and limited differentiation and development of multinucleated muscle cells was obtained. Normal human sera were essentially non-toxic to the human foetal muscle cultures, while 3 of 6 myasthenic sera gave significant myolysis. The limited nature of myotube development may have caused the extent of myolysis in this system to be underestimated, but again the high percentage of myolytic sera indicates the important contribution that muscle-specific membrane damage may have in the loss of available AChR observed in myasthenia gravis.

Suggestions for further study

In spite of the fact that some 90% of myasthenic patients have detectable anti-AChR antibody titres in their sera, the exact relationship of antibody to disease state is not clear. Although there is an overall statistical correlation when titre and clinical status are compared in sufficiently large groups of patients, the correlation is poor when individual patients are compared (Lefvert et al., 1978; Barkas et al., 1979). In myasthenia there is clearly a heterogeneous

population of antibodies (Mittag et al., 1981a,b) but not all of them are pathogenic, because high concentrations are known to occur in some patients in complete remission (Lefvert et al., 1978) and in healthy newborns to myasthenic mothers (Lefvert, 1978). Disease state may be more closely related to subpopulations of anti-receptor antibodies directed against particular determinants on the AChR. For this reason it is important to determine the antigenic specificities of anti-AChR antibodies in both myasthenia gravis and EAMG.

Antibodies are normally formed preferentially against the most exposed portions of the surface structure of an antigen (Crumpton 1974). When AChR is detergent-solubilised, the conformation of the molecule will be significantly altered from that of the membrane-bound receptor, and antigenic determinants normally hidden within the postsynaptic membrane may become exposed. Conversely, normally exposed sites may become masked. A number of reports have identified such differences in both ligand binding to the AChR (Boulter and Patrick, 1979) and in anti-receptor antibody reactivity (Martinez-Carrion et al., 1981; Souroujon et al., 1983; Neumann et al., 1984). The reaction of antibody with membrane-bound AChR should, therefore, be further studied in order to determine the antibody subpopulations which may directly affect muscle AChR function in myasthenia gravis and EAMG. In this respect the cross-reactive binding of antibodies raised against Torpedo AChR with chick muscle AChR in culture may represent a good model. A cross-reactivity between the foreign immunising AChR (from fish electric organ) and self muscle receptor is required for the induction of the autoimmune process in EAMG. It is thus important to study those antibodies that cross-react with muscle receptor. In view of the limited cross-reactivity between AChR from fish and skeletal muscle it is reasonable to suppose that cross-reactive antibodies will

be directed at highly conserved sites on the receptor. In so far as it is reasonable to assume that the conserved sites are relatively important in the functioning of the receptor molecule, then antibodies directed towards those sites could represent subclasses with particular relevance to the disease state in myasthenia gravis. Thus antibodies directed against the highly- conserved α- toxin binding site were found to be present predominantly in severely-ill myasthenic patients (Lefvert et al., 1981), and Lindstrom et al. (1981) found a homologous region on fish AChR and muscle AChR from a variety of sources which was highly immunogenic. The apparent homogeneity of binding of anti - (Torpedo AChR) antibody to chick myotubes found in this study (see preceding discussion) may also reflect well the situation in myasthenia gravis and EAMG. Pressman (1970) showed that in many cases the antibody population of an antiserum is composed of a limited number of antibody types with different affinities. Tzartos and Lindstrom (1980) detected a "main immunogenic region" on the extracellular surface of the α- subunit of AChRs from a number of species to which most of the antibodies in the sera of an animal immunised with intact AChR were directed, and Bray and Drachman (1982) found that Scatchard plots of myasthenic sera binding to human AChR were linear, indicating a dominant antibody type in the sera. Vincent and Newsom-Davis (1982) also demonstrated a population of high avidity antibody in each of 35 sera from myasthenic patients with ocular or generalised disease. Thus the specificity of binding to skeletal muscle in culture of both monoclonal and polyclonal antibodies raised against purified AChR should be extensively studied.

In this study antibody - mediated muscle cell lysis has been demonstrated to be a function of many myasthenic sera. The nature of the antibodies producing this lytic activity, and the role of

complement in producing myolysis need much further investigation. In particular the relationship of myolysis to the IgG subclass distribution in individual patients should be studied, in view of the wide variation in such distributions already demonstrated (Vincent and Bilkhu, 1982) and the known differences of the subclasses in their ability to fix complement (Natvig and Kunkel, 1973). The ability of myasthenic sera to activate complement irrespective of subclass distribution may also prove to be of significance, in light of the recent report of Adler et al. (1984) that autoantibodies to thyroglobulin inexplicably did not activate complement. The characteristics of anti-skeletal muscle antibodies other than anti-AChR which are present in many myasthenic sera (Gilhus et al., 1983), and their contribution to myolysis should also receive further study. The use of human muscle in culture may prove extremely useful in estimating the size of the contribution played by myolysis to the aetiology of myasthenia gravis. The development of mature cultures of relatively pure myotubes from human foetal material is therefore an important goal of future research. The use of serum-free defined media, and muscle - specific growth factors in achieving this aim are obvious areas for further study. Fambrough et al. (1982) have reported producing myotube cultures in which greater than 97% of nuclei were in multinucleated structures, lysing fibroblasts and myoblasts with a mAb specific for these cells, in conjunction with guinea pig complement. The use of such specific cytolytic agents should therefore also be further developed.

Myasthenia gravis still eludes a simplistic explanation, with a number of mechanisms contributing to the antibody-induced loss of functional AChR. As most patients have antibodies capable of inducing these pathological mechanisms in large excess over their AChR content,

endogenous factors must also be important in determining a patient's response to the immune assault. Attempts to understand, treat and cure the disease have led to significant advances in our knowledge of the AChR and of the underlying mechanisms of autoimmune diseases. This trend seems likely to continue.

REFERENCES

AARLI, J.A. (1970) Clin. Exp. Immunol. 7, 23-30.

ABDOU, N.I.,LISAK, R.P. and ZWEIMAN, B. (1974) New Engl, J. Med. 291, 1271-1275.

ADLER, T.R., BEALL, G.N., CURD, J.G., HEINER, D.C. and SABHARWAL, U.K. (1984) Clin. Exp. Immunol. 56, 383-389.

AHARONOV, A., TARRAB-HAZDAI, R., ABRANSKY, O. and FUCHS, S. (1975) Proc. Natl. Acad. Sci. USA 72, 1456-1459.

AHARONOV, A., TARRAB-HAZDAI, R., SILMAN, I. and FUCHS, S. (1977) Immunochem. 14, 129-137.

ALAJOUANINE, T., SCHERRER, J. and BOURGUIGNON, A. (1959) Revue Neurologique 100, 238-241.

ALMON, R.R. and APPEL, S.H. (1975) J. Neurochem. 27, 1513-1519.

ALMON, R.R., ANDREW, C.G. and APPEL, S.H. (1974) Science 186, 55-57

ANDERSON, D.J. and BLOBEL, G. (1981) Proc. Natl. Acad. Sci. USA 78, 5598-5602.

ANDERSON, D.J. and BLOBEL, G. (1983) Cold Spring Harbor Symp. Quant. Biol. 48, 125-134.

ANDREASON, J.J., DOERGE, D.R. and McNAMEE, M.G. (1979) Arch. Biochem. Biophys. 194, 468-480.

ANHOLT, R. (1981) Trends in Biochem. Sci. 6, 288-291.

ANHOLT, R., FREDKIN, D.R., DEERINCK, T., ELLISMAN, M., MONTAL, M. and LINDSTROM, J. (1982) J. Biol. Chem. 257, 7122-7134.

APPEL, S.H., ALMON, R.R. and LEVY, N. (1975) New Engl. J. Med. 293, 760-761.

APPEL, S.H., ANWYL, R., McADAMS, M.W. and ELIAS, S. (1977) Proc. Natl. Acad. Sci. USA 74, 2130-2134.

ARGOV, Z., NICHOLSON, L., FAWCETT, P.R.W., MASTAGLIA, F.L. and HALL, M. (1980) Lancet 1, 203.

ARNON, R. (1973) in The Antigens (SELA, M. ed.) Vol.1, p.87, Academic Press, New York.

ATASSI, M.Z. (1975) Immunochem. 12, 423-438.

ATASSI, M.Z. (1978) Immunochem. 15, 909-936.

AXELROD, D., RAVDIN, P., KOPPEL, D.E., SCHLESSINGER, J., WEBB, W.W., ELSON, E.L. and PODLESKI, T.R. (1976) Proc. Natl. Acad. Sci. USA 73, 4594-4598.

BALLIVET, M., PATRICK, J., LEE, J. and HEINEMANN, S. (1982) Proc. Natl. Acad. Sci. USA 79, 4466-4470.

BARKAS, T., HARRISON, R., LUNT, G.G. and WATSON, C.M.J. (1978) Biochem. Soc. Trans. 6, 636-638.

BARKAS, T., HARRISON, R., LUNT, G.G., STEPHENSON, F.A., BEHAN, P.O. and SIMPSON, J.A. (1979) in Progress in Neurological Research (BEHAN, P.O. and CLIFFORD ROSE F. eds.) pp.169-174, Pitman Medical Press, Tunbridge Wells.

BARNARD, E.A., DOLLY, J.O., LO, M. and MANTLE, T. (1978) Biochem. Soc. Trans. 6, 649-651.

BARNARD, E.A., DOLLY, J.O., LANG, B., LO, M. and SHORR, R.G. (1979) in Advances in Cytopharmacology (Ceccarelli, B. and Clementi, F. eds.) Vol.3, pp.409-436, Raven Press, New York.

BARNSTAPLE, C., JESSELL, T., SANES, J., STEVENS, C. and ROBERTSON, M. (1983) Nature 306, 14-16.

BARRANTES, F.J., NEUGEBAUER, D.-CH. and ZINGSHEIM, H.P. (1980) FEBS Lett. 112, 73-78.

BARTFIELD, D., and FUCHS, S. (1977) FEBS Lett. 77, 214-218.

BARTFIELD, D. and FUCHS, S. (1979) Biochem. Biophys. Res. Commun. 89, 512-519.

BAUMANN, H. and DOYLE, D. (1980) Cell 21, 897-907.

BENDER, A.N., ENGEL, W.K., RINGEL, S.P., DANIELS, M.P. and VOGEL, Z. (1975) Lancet 1, 607-608.

BERANEK, R. and VYSKOCIL, F. (1967) J. Physiol. 188, 53-66.

BERG, D.K., KELLY, R.B., SARGENT, P.B., WILLIAMSON, P. and HALL, Z.W. (1972) Proc. Natl. Acad. Sci. USA 69, 147-151.

BERGSTROM, K., LEFVERT, A.K., MATELL, G. and PIRKANEN, R. (1978) in The Biochemistry of Myasthenia Gravis and Muscular Dystrophy (LUNT, G.G. and MARCHBANKS, R.M. eds.) pp.177-178, Academic Press, London and New York.

BERMAN, P.W. and HEINEMANN, S.F. (1984) J. Immunol. 132, 711-717.

BERMAN, P.W., PATRICK, J., HEINEMANN, S., KLIER, F.G. and STEINBACH, J.H. (1981) Ann. N.Y. Acad. Sci. 377, 237-256.

BERSINGER, N.A., VELAZCO, M.-I., JAMES, R.W. and FULPIUS, B.W. (1983) Neurochem. Int. 5, 459-462.

BEUTNER, E.N., WITEBSKY, E., RICKEN, D. and ADLER, R.H. (1962) J. Am. Med. Assoc. 182, 46-58.

BEVAN, S. and STEINBACH, J.H. (1977) J. Physiol. (London) 267, 195-213.

BEVAN, S., KULLBERG, R.W. and HEINEMANN, S.F. (1977) Nature 267, 263-265.

BEVAN, S., KULLBERG, R.W. and RICE, J. (1978) Nature 273, 469-471.

BIESECKER, G. (1973) Biochem. 12, 4403-4409.

BISCHOFF, R. (1975) The Anatomical Record 182, 215-236.

BLANCHARD, S.G., QUAST, U., REED, K., LEE, T., SCHIMERLIK, I., VANDLEN, R., CLAUDIO, T., STRADER, C.D., MOORE, H-P.H. and RAFTERY, M.A. (1979) Biochem. 18, 1875-1883.

BLOCH, R.J. (1979) J. Cell. Biol. 82, 626-643.

BLOCH, R.J. and GEIGER, B. (1980) Cell 21, 25-35.

BLUMCKE, S. and NIEDORF, H.R. (1965) Virchow's Archiv. fur Pathologisch, Anatomie und Physiologie 340, 93-104.

BOULTER, J. and PATRICK, J. (1979) J. Biol. Chem. 254, 5652-5657.

BRADLEY, R.J., DWYER, D., MORLEY, B.J., ROBINSON, G., KEMP, G.E. and OH, S.J. (1979) Prog. Brain Res. 49, 441-448.

BRAY, J.J. and DRACHMAN, D.B. (1982) J. Immunol. 128, 105-110.

BROCKES, J.P. and HALL, Z.W. (1975) Biochem. 14, 2092-2099.

BURDEN, S. (1977) Dev. Biol. 61, 79-85.

BURSZTAJN, S. and FISHBACH, G.D. (1984) J. Cell Biol. 98, 498-506.

CAMBRIDGE, G. and STERN, C.M.M. (1981) Clin. Exp. Immunol. 43, 211-219.

CARTER, B., HARRISON, R., LUNT, G.G., BEHAN, P.O. and SIMPSON, J.A. (1980) J. Neurol. Neurosurg. Psychiat. 43, 397-402.

CARTER, B., HARRISON, R., LUNT, G.G., MORRIS, H., SAVAGE-MARENGO, T. and STEPHENSON, F.A. (1981) Ann. Clin. Biochem. 18, 146-152.

CASTLEMAN, B. (1966) Ann. N.Y. Acad. Sci. 135, 496-505.

CHABNER, B.A. (1981) in Cancer and Chemotherapy Vol. III (CROOKE, S.T. and PRESTAYKO, A.W. eds.) pp.3-24, Academic Press, London.

CHANG, H.W. and BOCK, E. (1979) Biochem. 18, 172-179.

CHANGEUX, J.P. (1975) in Handbook of Psychopharmacology (IVERSON, L.L. ed.) Vol.6, pp.235-301, Plenum Press, New York.

CHANGEUX, J.P. (1981) in The Harvey Lectures, Series 75, pp.85-254, Academic Press, New York.

CHANGEUX, J.P. and DANCHIN, A. (1976) Nature 264, 705-712.

CHANGEUX, J.P., GAUTRON, J., ISRAEL, M. and PODLESKI, T. (1969) Comp. Rend. Acad. Sci. Paris 269, 1788-1791.

CHANGEUX, J.P., KASAI, M. and LEE, C.Y. (1970) Proc. Natl. Acad. Sci. USA 67, 1241-1247.

CHANGEUX, J.P., MEUNIER, J.C. and HUCHET, M. (1971) Mol. Pharmacol. 5, 538-553.

CINADER, B. (1963) Ann. N.Y. Acad. Sci. 103, 495.

CLAUDIO, T.R. and RAFTERY, M.A. (1980) J. Immunol. 124, 1130.

CLEATOR, G.M. and BESWICK, T.S.L. (1972) Cytobios 5, 231-239.

COERS, C. and WOOLF, A.L. (1959) The Innervation of Muscle: A Biopsy study. Blackwell Scientific Publications, Oxford.

COHEN, E.P. and LIANG, W. (1976) in Membranes and Neoplasia: New Approaches and Strategies (MARCHESI, V.T. ed.) pp.89-98, Liss, New York.

COHEN, J.B., BOYD, N.D., YENG, A.Y., MEDYNSKI, D.C., NEUBIG, R.R, St. JOHN, P. and SHERA, N.S. (1980) in Psychopharmacology and Biochemistry of Neurotransmitter Receptors (YAMAMURA, H., OLSEN, R. and USDIN, eds.) pp.47-62, Elsevier/North Holland, Amsterdam.

COLQUHOUN, D. (1979) in The Receptors. A Comprehensive Treatise (O'BRIEN, R.D. ed.) Vol.1, pp.93-142, Plenum Press, New York.

COMPSTON, D.A.S., VINCENT, A., NEWSOM-DAVIS, J. and BATCHELOR, J.R. (1980) Brain 103, 579-601.

CONTI-TRONCONI, B.M. and RAFTERY, M.A. (1982) Ann. Rev. Biochem. 51, 491-530.

CONTI-TRONCONI, B.M., DUNN, S.M.J. and RAFTERY, M.A. (1982) Biochem. 21, 893-899.

COOPER, D. and REICH, E. (1972) J. Biol. Chem. 247, 3008-3013.

CRAIN, J.M., ALFEI, L. and PETERSON, E.R. (1970) J. Neurobiol. 1, 471-489.

CRIADO, M. AND BARRANTES, F.J. (1982) Neurochem. Int. 4, 289-302.

CRIADO, M., EIBL, H. and BARRANTES, F.J. (1984) J. Biol. Chem. 259, 9188-9198.

CRUMPTON, (1974) in The Antigens (SELA, M. ed.) Vol.2, p.173, Academic Press, New York.

CULL-CANDY, S.G., MILEDI, R., TRAUTMANN, A. and UCHITEL, O.D. (1980) J. Physiol. 299, 621-638.

CURRIE, S. (1970) Acta Neuropathol. (Berlin) 15, 11.

DALE, H.H. (1914) J. Pharmacol. Exp. Ther. 6, 147-190.

DALE, H.H., FELDBERG, W. and VOGT, M. (1936) J. Physiol. (London) 86, 353-380.

DALY, J.W., KARLE, I., MYERS, C.W., TOKUYAMA, T., WATERS, J.A. and WITKOP, B. (1971) Proc. Natl Acad Sci. USA 68, 1870.

DALZIEL, A.W., ROLLINS, E.S. and McNAMEE, M.G. (1980) FEBS Lett. 122, 193-196.

DAWKINS, R.L. and MASTAGLIA, F.L. (1973) New Engl. J. Med. 288, 434-438.

DE LA HABA, G. and AMUNDSEN, R. (1972) Proc. Natl. Acad. Sci. USA 69, 1131-1135.

DEL CASTILLO, J. and KATZ, B. (1955) J. Physiol. 128, 157-181.

DE ROBERTIS, E., FISZER, S. and SOTO, E.F. (1967) Science 158, 928.

DESMEDT, J.E. (1966) Ann. N.Y. Acad. Sci. 135, 209-246.

DESOUKI, A., ELDEFRAWI, A.T. and ELDEFRAWI, M.E. (1981) Exp. Neurol. 73, 440-450.

DEVILLERS-THIERY, A., CHANGEUX, J.P., PAROUTAUD, P. and STRASBERG, A.D. (1979) FEBS Lett. 104, 99-105.

DEVILLERS-THIERY, A., GIRAUDAT, J., BENTABOULET, M. and CHANGEUX, J.P. (1983) Proc. Natl. Acad. Sci. USA 80, 2067-2071.

DEVREOTES, P.N. and FAMBROUGH, D.M. (1975) J. Cell Biol. 65, 335.

DEVREOTES, P.N. and FAMBROUGH, D.M. (1976) Cold Spring Harbor Symp Quant. Biol. 40, 237.

DIXON, W.E. (1906) Brit. Med. J. 2, 1807.

DOLLY, J.O. (1979) in Physiological and Pharmacological Biochemistry (TIPTON, K. ed.) Vol.26, pp.257-309, University Park Press, Baltimore.

DOLLY, J.O. and BARNARD, E.A. (1974) FEBS Lett. 46, 145-149.

DOLLY, J.O. and BARNARD, E.A. (1977) Biochem. 16, 5053-5060.

DOWNES, J.M., GREENWOOD, B.M. and WRAY, S.H. (1966) Quarterly J. Med. 35, 85-105.

DRACHMAN, D.B. (1981) Ann. Rev. Neurosci. 4, 195-225.

DRACHMAN, D.B., ANGUS, C., ADAMS, R., MICHELSON, J. and HOFFMAN, G. (1978) New Engl. J. Med. 298, 1116-1122.

DRACHMAN, D.B., ADAMS, R.V., JOSIFEK, L.F. and SELF, S.G. (1982) New Engl. J. Med. 307, 769-775.

DRYDEN, W.F. (1970) Experientia 26, 984-986.

DU BOIS RAYMOND (1877) in Gessammelte Abhandlungen zur allgemeinen Muskel-und Nervenphysik Vol.2, Veit, Leipzig.

DU VERNAY, V.H. (1981) in Cancer and Chemotherapy VolIII (CROOKE, S.T. and PRESTAYKO, A.W. eds.) pp.234-271, Academic Press, London.

DWYER, D.S., BRADLEY, R.J., OH, S.J. and KEMP, G.E. (1979) Clin. Exp. Immunol. 37, 448-451.

DWYER, D.S., BRADLEY, R.J., FURNER, R.L. and KEMP, G.E. (1981a) Brain Res. 217, 23-40.

DWYER, D.S., KEARNEY, J.F., BRADLEY, R.J., KEMP, G.E. and OH, S.J. (1981b) Ann. N.Y. Acad. Sci. 377, 143-155.

EDELMAN, G.M. and GALL, W.E. (1969) Ann. Rev. Biochem. 38, 415-466.

EHRMANN, R.L. and GEY, G.O. (1956) J. Natl. Cancer Inst. 16, 1375-1403

EINARSON, B., GULLICK, W., CONTI-TRONCONI, B., ELLISMAN, M. and LINDSTROM, J. (1982) Biochem. 21, 5295-5302.

EISEN, H. (1974) Immunology pp.373-403, Harper and Row, Hagerstown, Maryland.

EKSTEDT, J. and STALBERG, E. (1967) Opuscula Medica 12, 73-76.

ELDEFRAWI, M.E. (1978) Fed. Proc. 37, 2823-2827.

ELDEFRAWI, M.E. and ELDEFRAWI, A.T. (1973a) Biochem. Pharmacol. 22, 3145-3150.

ELDEFRAWI, M.E. and Eldefrawi, A.T. (1973b) Arch. Biochem. Biophys. 159, 362-373.

ELDEFRAWI, M.E., ELDEFRAWI, A.T., SEIFERT, S. and O'BRIEN, R.D. (1972) Arch. Biochem. Biophys. 150, 210- 218.

ELLENA, J.F., BLAZING, M.A. and McNAMEE, M.G. (1983) Biochem. 22, 5523-5535.

ELMQVIST, D., HOFMANN, W.W., KUGELBERG, J. and QUASTEL, D.M.J. (1964) J. Physiol. 174, 417-434.

ELSON, H.F. (1979) J. Supramol. Struc. 10, 39-50.

EMERY, A.E.H. and McGREGOR, L. (1977) Clin. Genetics 12, 183-187.

ENGEL, A.G., TSUJIHATA, M., LAMBERT, E., LINDSTROM, J. and LENNON, V.A. (1976a) J. Neuropath. Exp. Neurol. 35, 569-587.

ENGEL, A.G., TSUJIHATA, M., LINDSTROM, J. and LENNON, V.A. (1976b) Ann. N.Y. Acad. Sci. 274, 60-79.

ENGEL, A.G., LINDSTROM, J.M., LAMBERT, E.H. and LENNON, V.A. (1977) Neurol. 27, 307-315.

ENGEL, A.G., SAKAKIBARA, H., SAHASHI, K., LINDSTROM, J., LAMBERT, E. and LENNON, V. (1979) Neurol. 29, 179.

ENGEL, A.G., SAHASHI, K. and FUMAGALLI, G. (1981) Ann. N.Y. Acad. Sci. 377, 158-173.

EPSTEIN, M. and RACKER, E. (1978) J. Biol. Chem. 253, 6660-6662.

FAMBROUGH, D.M. (1979) Physiol. Rev. 59, 165-227.

FAMBROUGH, D.M. (1981) in Receptors and Recognition Series B (LEFKOWITZ, R.J. ed.) Vol.13, pp.125-142.

FAMBROUGH, D.M. (1984) in Neuromuscular Diseases (SERRATRICE, G. et al., eds.) pp.465-470, Raven Press, New York.

FAMBROUGH, D.M. and RASH, J.E. (1971) Dev. Biol. 26, 55-63.

FAMBROUGH, D.M. and DEVREOTES, P.N. (1978) J. Cell Biol. 76, 237-244.

FAMBROUGH, D.M., BAYNE, E.K., GARDNER, J.M., ANDERSON, M.J., WAKSHULL, E. and ROTUNDO, R.L. (1982) in Neuroimmunology (BROCKES, J. ed.) pp.49-90, Plenum Press, New York.

FATT, P. and KATZ, B. (1951) J. Physiol. 115, 320-370.

FELTKAMP, T.W., VAN DEN BERG-LOONEN, P.M., NIJENHUIS, L.E., ENGELFRIET, C.P., VAN ROSSUM, A.L., VAN LOGHEM, J.J. and OOSTERHUIS, H.J.G.H. (1974) Br. Med. J. 1, 131-133.

FISCHBACH, G.D. (1972) Dev. Biol. 28, 407.

FISCHBACH, G.D. and COHEN, S.A. (1973) Dev. Biol. 31, 147-162.

FROEHNER, S.C., REINESS, C.G. and HALL, Z.W. (1977a) J. Biol. Chem. 252, 8589-8596.

FROEHNER, S.C., KARLIN, A. and HALL, Z.W. (1977b) Proc. Natl. Acad. Sci. USA 74, 4685-4688.

FUCHS, S., BARTFIELD, D., MOCHLY-ROSEN, D., SOUROUJON, M. and FEINGOLD, C. (1981) Ann. N.Y. Acad. Sci. 377, 110-123.

FULPIUS, B.W. (1976) in Motor Innervation of Muscle (THESLEFF, S. ed.) pp.1-29, Academic Press, London and New York.

FULPIUS, B.W., ALLIOD, C., BERSINGER, N.A., GUENOUD, S., JAMES, R.W. and ZURN, A.D. (1978) Muscle and Nerve 1, 336.

FULPIUS, B.W., MISKIN, R. and REICH, E. (1980) Proc. Natl. Acad. Sci. USA 77, 4326-4330.

GILHUS, N.E., AARLI, J.A. and MATRE, R. (1983) J. Neuroimmunol. 5, 239-249.

GOLDSTEIN, G. and MANGANARO, A. (1971) Ann. N.Y. Acad. Sci. 183, 230-240.

GOLDSTEIN, G. and WHITTINGHAM, S. (1966) Lancet ii, 315-318.

GOLDSTEIN, A.L., THURMAN, G.B., COHEN, G.H. and ROSSIO, J.L. (1976) Ann. N.Y. Acad. Sci. 274, 390-401.

GOMEZ, C.M. and RICHMAN, D.P. (1983) Proc. Natl. Acad. Sci. USA

FRANKLIN, G I, YASIN, R, HUGHES, B P and THOMPSON, EJ (1980) J Neurol Sci 43, 317-327.

30, 4089-4093.

GOMEZ, C.M., RICHMAN, D.P., BERMAN, P.W., BURRES, S.A., ARNASON, B.G.W. and FITCH, F.W. (1979) Biochem. Biophys. Res. Commun. 88, 575-582.

GORDON, A.S., MILFAY, D. and DIAMOND, I. (1983) Proc. Natl. Acad. Sci. USA 80, 5862-5866.

GOTTI, C., CONTI-TRONCONI, B.M. and RAFTERY, M.A. (1982) Biochem. 21, 3148-3154.

GOTTI, C., CASADEI, G.L. and CLEMENTI, F. (1983) Neurosci. Lett. 35, 143-148.

GREVILLE, G.D. and TUBBS, P.K. (1968) Essays in Biochem. 4, 155.

GULLICK, W.J. and LINDSTROM, J.M. (1982) J. Cell Biochem. 19, 223-230.

GULLICK, W., TZARTOS, S. and LINDSTROM, J. (1981) Biochem. 20, 2173-2180.

HALL, J.E. (1981) Biophys. J. 33, 373-381.

HALL, Z.W., ROISIN, M.P., GORIN, P. and GU, Y. (1983) Proc.of Int. Union of Physiol. Sci. 15, 383 (Abstract).

HANLEY, M.R. (1978) Biochem. Biophys. Res. Commun. 82, 392-401.

HARRISON, R. and LUNT, G.G. (1980) Biological Membranes:Their Structure and Function. pp.131-165, Blackie, Glasgow.

HARRISON, R., LUNT, G.G., MORRIS, H. and SAVAGE-MARENGO, T. (1981) Ann. N.Y. Acad. Sci. 377, 332-341.

HARVEY, A.L. and DRYDEN, W.F. (1974) Eur. J. Pharmacol. 27, 5-13.

HARVEY, A.L., BARKAS, T., HARRISON, R. and LUNT, G.G. (1978) in The Biochemistry of Myasthenia Gravis and Muscular Dystrophy (LUNT, G.G. and MARCHBANKS, R.M. eds.) pp.167-175, Academic Press, London.

HARVEY, A.L., ROBERTSON, J.G., BARKAS, T., HARRISON, R., LUNT, G.G., STEPHENSON, F.A., CAMPBELL, M.J. and TEAGUE, R.H. (1978) Clin. Exp. Immunol. 34, 411-416.

HASSON-VOLOCH, A. (1968) Nature 218, 330-333.

HAUSHKA, S.D. (1972) in Growth, Nutrition and Metabolism of Cells in Culture Vol. 2 (ROTHBLAT, G.H. and CRISTOFALO, V.J. eds.) pp.68-130, Academic Press, New York.

HAUSHKA, S.D. (1974a) Dev. Biol. 37, 329-344.

HAUSHKA, S.D. (1974b) Dev. Biol. 37, 345-368.

HAUSHKA, S.D. and KONIGSBERG, I.R. (1966) Proc. Natl. Acad. Sci. USA 55, 119.

HEIDMANN, T. and CHANGEUX, J.P. (1978) Ann. Rev. Biochem. 47, 317-357.

HEIDMANN, T., SOBEL, A. and CHANGEUX, J.P. (1980) Biochem. Biophys. Res. Commun. 93, 127-133.

HEILBRONN, E., MATTSON, C. and ELFMAN, L. (1974) Proc.9th FEBS meeting 37, 29-37.

HEINEMANN, S., BEVAN, S., KULLBERG, R., LINDSTROM, J. and RICE, J. (1977) Proc. Natl. Acad. Sci. USA 74, 3090-3094.

HEINEMANN, S., MERLIE, J.P. and LINDSTROM, J. (1978) Nature (London) 274, 65-67.

HUANG, L. (1979) FEBS Lett. 102, 9-12.

HUCHO, F. (1981) Trends in Biochem. Sci. 6, 242-245.

HUDSON, L. and HAY, F.C. (1976) Practical Immunology, Blackwell Scientific Publications, Oxford.

HUNTER, W.M. (1978) in Handbook of Experimental Immunology (WEIR, D.M. ed.) pp.14.1-14.40, Blackwell, Oxford.

HUNTER, W.M. and GREENWOOD, F.C. (1962) Nature (London) 194, 495-496.

ITO, Y., MILEDI, R., MOLENAAR, P.C., VINCENT, A., POLAK, R.L., VAN GELDER, M. and DAVIS, J.N. (1976) Proc. Roy. Soc. 192, 475-480.

ITO, Y., MILEDI, R., VINCENT, A. and NEWSOM-DAVIS, J. (1978) Brain 101, 345-368.

JAILKHANI, B.L., ASTHANA, D., JAFFERY, N.F. and SUBBALAXMI, B. (1984) J. Neuroimmunol. 6, 337-345.

JAMES, R.W., BERSINGER, N.A., SCHWENDIMANN, B. and FULPIUS, B.W. (1980) Hoppe-Seyler's Z. Physiol. Chem. 361, 1517-1524.

JAMES, R.W., ALLIOD, C. and FULPIUS, B.W. (1983) Mol. Immunol. 20, 1363-1368.

JOHNS, T.R., CROWLEY, W.J., MILLER, J.Q. and CAMPA, J.F. (1971) Ann. N.Y. Acad. Sci. 183, 64-71.

JOHNSON, I.S. (1982) in Cancer Medicine (HOLLAND, J.F. and FREI, E. eds.) pp.910-920, Lea and Febiger, Philadelphia.

JOHNSON, A.G. and WOOLF, A.L. (1965) Acta Neuropathalogica 4, 436-441.

JOLLY, F. (1895) Berliner Klinische Wochenschrift 32, 1-7.

KAKULAS, B.A., PAPADIMITRIOU, J.M., KNIGHT, J.O. and MASTAGLIA, F.L. (1968) Proc. Aust. Assoc. Neurol. 5, 79-85.

KAO, I. and DRACHMAN, D.B. (1977) Science 195, 74-75.

KARLIN, A. (1974) Life Sci. 14, 1385-1415.

KARLIN, A. (1980) in The Cell Surface and Neuronal Function (POSTE, G., NICOLSON, G. and COTMAN, C. eds.) pp.191-260, Elsevier-North Holland Biomedical Press, New York.

KARLIN, A., PRIVES, J., DEAL, W. and WINNIK, M. (1971) J. Mol. Biol. 61, 175-188.

KARLIN, A., HOLTZMAN, E., VALDERRAMA, R., DAMLE, V., HSU, K. and REYES, F. (1978) J. Cell Biol. 76, 577-592.

KARLIN, A., DAMLE, V., HAMILTON, S., McLAUGHLIN, M., VALDERRAMA, P. and WISE, D. (1979) in Advances in Cytopharmacology (CECCARELLI, B. and CLEMENTI, F. eds.) Vol.3, pp.183-188, Raven Press, New York.

KARLSSON, E., HEILBRONN, E. and WIDLUND, L. (1972) FEBS Lett. 28, 107-111.

KATZ, B. and MILEDI, R. (1965) Proc. Roy. Soc. (London) Series B. 161, 453-495.

KATZ, B. and MILEDI, R. (1972) J. Physiol. 224, 665-669.

KEESEY, J.C., TOURTELLOTE, W.W., HERMANN, C., ANDREWS, J.M. and LINDSTROM, J. (1978) Lancet i, 777.

KEMP, G., MORLEY, B., DWYER, D. and BRADLEY, R.J. (1980) Membrane Biochem. 3, 229-257.

KISTLER, J., STROUD, R.M., KLYMKOWSKY, M.W., LALANCETTE, R.A. and FAIRCLOUGH, R.H. (1982) Biophys. J. 37, 371-383.

KLETT, R.P., FULPIUS, B.W., COOPER, D., SMITH, M., REICH, E. and POSSANI, L.D. (1973) J. Biol. Chem. 248, 6841-6853.

KOETHE, S.M., COOK, A., McQUILLEN, D.P. and McQUILLEN, M.P. (1981) Ann. N.Y. Acad. Sci. 377, 447-453.

KOTT, E. and RULE, A.H. (1973) Neurology (Minneap.) 23, 745-765.

KROHN, K., SHERMAN, L. and WELCH, M. (1972) Biochim. Biophys. Acta 285, 404-413.

LANGLEY, J.N. (1907) J. Physiol. (London) 36, 347-384.

LAYNE, E. (1957) Methods in Enzymology 3, 450-451.

LEE, C.Y. (1972) Ann. Rev. Pharmacol. 12, 265-286.

LEE, C.Y. and CHANG, C.C. (1966) Mem. Inst. Butantan. Internac. 33, 555-572.

LEFVERT, A.K. (1978) Clin. Exp. Immunol. 34, 111-117.

LEFVERT, A.K. (1981) Ann. N.Y. Acad. Sci. 377, 125-141.

LEFVERT, A.K. (1982) J. Neurol. Neurosurg. Psychiatry 45, 70-73.

LEFVERT, A.K. and BERGSTROM, K. (1977) Eur. J. Clin. Invest. 7, 115-119.

LEFVERT, A.K., BERGSTROM, K., MATELL, G., OSTERMAN, P.O. and PIRSKANEN, R. (1978) J. Neurol. Neurosurg. Psychiatry 41, 1-10.

LEFVERT, A.K., CUENOUD, S. and FULPIUS, B.W. (1981) J. Neuroimmunol. 1, 125-135.

LENNON, V.A. (1976) Immunol. Commun. 5, 323-344.

LENNON, V.A. and LAMBERT, E.H. (1980) Nature 285, 238-240.

LENNON, V.A. and LAMBERT, E.H. (1981) Ann. N.Y. Acad. Sci. 377, 77-95.

LENNON, V.A., LINDSTROM, J.M. and SEYBOLD, M.E. (1975) J.Exp. Med. 141, 1365-1375.

LENNON, V.A., SEYBOLD, M.E., LINDSTROM, J., COCHRANE, C. and YULEVITCH, R. (1978)J. Exp. Med. 147, 973-983.

LESTER, H. (1977) Sci. Amer. 236, 106-118.

LEVINE, G.D. and RICE, D.F. (1977) Lab. Invest. 36, 345.

LEWIS, M.R. and LEWIS, W.H. (1917) Am. J. Anat. 22, 169-194.

LINDSTROM, J. (1977) J. Clin. Immunol. Immunopathol. 7, 36-73.

LINDSTROM, J. (1978) Fed. Proc. 37, 2828-2830.

LINDSTROM, J. (1979) Adv. Immunol. 27, 1-49.

LINDSTROM, J. and EINARSON, B. (1979) Muscle and Nerve 2, 173-179.

LINDSTROM, J.M. and ENGEL, A. (1981) in Receptors and Recognition Series B (CUATRECASAS, P. and GREAVES, M. eds.) pp.161-214, Chapman and Hall.

LINDSTROM, J. and LAMBERT, E. (1978) Neurol. 28, 130-138.

LINDSTROM, J. and PATRICK, J. (1974) in Synaptic Transmission and Neuronal Interaction (BENNET, M.V.L. ed.) pp.191-216, Raven Press, New York.

LINDSTROM, J., EINARSON, B., LENNON, V.A. and SEYBOLD, M.E. (1976a) J. Exp. Med. 144, 726-738.

LINDSTROM, J., ENGEL, A.G., SEYBOLD, M.E., LENNON, V.A. and LAMBERT, E.H. (1976b) J. Exp. Med. 144, 739-753.

LINDSTROM, J.M., LENNON, V., SEYBOLD, M.E. and WHITTINGHAM, S. (1976c) Ann. N.Y. Acad. Sci. 274, 254-274.

LINDSTROM, J.M., SEYBOLD, M.E., LENNON, V.A., WHITTINGHAM, S. and DUANE, D.D. (1976d) Neurol. 26, 1054-1059.

LINDSTROM, J., EINARSON, B. and FRANCY, M. (1977) in Cellular Neurobiology (HALL, Z. and KELLEY, R. eds.) pp.119-130, Liss, New York.

LINDSTROM, J., EINARSON, B. and MERLIE, J. (1978) Proc. Natl. Acad. Sci. USA 75, 769-773.

LINDSTROM, J., MERLIE, J. and YOGEESWARAN, G. (1979a) Biochem. 18, 4465-4470.

LINDSTROM, J., WALTER, B. and EINARSON, B. (1979b) Biochem. 18, 4470-4480.

LINDSTROM, J., GULLICK, W., CONTI-TRONCONI, B. and ELLISMAN, M. (1980) Biochem. 19, 4791-4795.

LINDSTROM, J., TZARTOS, S. and GULLICK, W. (1981) Ann. N.Y. Acad. Sci. 377, 1-19.

LIVESON, J.A., GODMAN, G., KORNFIELD, P. and BORNSTEIN, M.B. (1976) Exp. Neurol. 50, 620-627.

LO, M.M.S., GARLAND, P.B., LAMPRECHT, J. and BARNARD, E.A. (1980) FEBS Lett. 111, 407-412.

LOEWI, O. (1921) Pfugers Arch. ges Physiol. 189, 239-242.

LOTWICK, H., HARRISON, R., LUNT, G. and BEHAN, P. (1983) J. Neuroimmunol. 4, 167-174.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J. (1951) J. Biol. Chem. 193, 265-275.

LUKASIEWICZ, R.J., HANLEY, M.R. and BENNETT, E.L. (1978) Biochem. 17, 2308-2313.

LYDDIAT, A., SUMIKAWA, K., WOLOSIN, J.M., DOLLY, J.O. and BARNARD, E.A. (1979) FEBS Lett. 108, 20-24.

MacDERMOTT, V. (1960) Brain 83, 24-36.

MARCH, S.C., PARIKH, I. and CUATRECASAS, P. (1974) Analyt. Biochem. 60, 145-152.

MARKELONIS, G.J. and OH, T.H. (1983) J. Neurochem. 4 (Suppl.) S108B.

MARKELONIS, G.J., KEMERER, V.F. and OH, T.H. (1980) J. Biol. Chem. 255, 8967-8970.

MARSH, D., WATTS, A. and BARRANTES, F.J. (1981) Biochim. Biophys. Acta 645, 97-101.

MARTINEZ, R.D., TARRAB-HAZDAI, R., AHARONOV, A. and FUCHS, S. (1977) J. Immunol. 118, 17-20.

MARTINEZ-CARRION, M., FARACH, M. and MIHOVILOVIC, M. (1981) Ann. N.Y. Acad. Sci. 377, 846-847.

MATTSON, C. and HEILBRONN, E. (1975) J. Neurochem. 25, 899-901.

MAURO, A. (1961) J. Biophys. Biochem. Cytol. 9, 493-495.

McCONAHEY, P.J. and DIXON, F.J. (1966) Int. Arch. Allergy 29, 185-189.

McNAMEE, M.G. and OCHOA, E.L.M. (1982) Neurosci. 7, 2305-2319.

McNAMEE, M.G., WEILL, C.L. and KARLIN, A. (1975) Ann. N.Y. Acad. Sci. 264, 175-181.

MEHL, V.S. and LANG, R.W. (1984) J. Neuroimmunol. 6, 347-360.

MERLIE, J.P. and SEBBANE, R. (1981) J. Biol. Chem. 256, 3605-3608.

MERLIE, J.P., SOBEL, A., CHANGEUX, J.P. and GROS, F. (1975) Proc. Natl. Acad. Sci. USA 72, 4028-4032.

MERLIE, J.P., HEINEMANN, S., EINARSON, B. and LINDSTROM, J. (1979) J. Biol. Chem. 254, 6328-6332.

MERLIE, J.P., HOFFLER, J. and SEBBANE, R. (1981) J. Biol. Chem. 256, 6995.

MERLIE, J.P., SEBBANE, R., TZARTOS, S. and LINDSTROM, J. (1982) J. Biol. Chem. 257, 2694-2701.

MERLIE, J.P., SEBBANE, R., GARDNER, S., OLSON, E. and LINDSTROM, J. (1983) Cold Spring Harbor Symp. Quant. Biol. 48, 135-146.

MEUNIER, J.C., OLSEN, R.W., MENEZ, A., MORGAT, J.L., FROMAGEOT, P., RONSERAY, A.M., BOQUET, P. and CHANGEUX, J.P. (1971) Comp. Rend. Acad. Sci. Paris 273, 595-598.

MEUNIER, J.C., OLSEN, R.W., MENEZ, A., FROMAGEOT, P., BOQUET, P. and CHANGEUX, J.P. (1972a) Biochem. 11, 1200-1210.

MEUNIER, J.C., OLSEN, R.W. and CHANGEUX, J.P. (1972b) FEBS Lett. 24, 63-68.

MEUNIER, J.C., SEALOCK, R., OLSEN, R.W. and CHANGEUX, J.P. (1974) Eur. J. Biochem. 45, 371-394.

MIHOVILOVIC, M. and MARTINEZ-CARRION, M. (1979) Biochem. 18, 4522-4528.

MILEDI, R., MOLINOFF, P. and POTTER, L.T. (1971) Nature 229, 554-557.

MINGUETTI, G. and MAIR, W.G.P. (1981) Biol. Neonate 40, 276-294.

MITTAG, T.W., KORNFIELD, P., TORMAY, A. and WOO, C. (1976) New Engl. J. Med. 294, 691-694.

MITTAG, T.W., TORMAY, A. and MASSA, A. (1978) Mol. Pharmacol. 14, 60-68.

MITTAG, T., MASSA, T., KORNFIELD, P., PAPATESTAS, A., BENDER, A. and GENKINS, G. (1981a) Ann. N.Y. Acad. Sci. 377, 342-357.

MITTAG, T., MASSA, T., KORNFIELD, P., PAPATESTAS, A., BENDER, A. and GENKINS, G. (1981b) Muscle and Nerve 4, 16-25.

MOCHLY-ROSEN, D., FUCHS, S. and ESHHAR, Z. (1979) FEBS Lett.106, 389-392.

MOMOI, M.Y. and LENNON, V.A. (1982) J. Biol. Chem. 257, 12757-12764.

MONNIER, V.M. and FULPIUS, B.W. (1977) Clin. Exp. Immunol. 29, 16-22.

MORGAN, J. and COHEN, L. (1974) In Vitro 10, 188-195.

MORRIS, G.E. and COLE, R.J. (1972) Exp. Cell Res. 75, 191-199.

MOSS, M., NORRIS, J.S., PECK, E.J. and SCHWARTZ, R.J. (1978) Exp. Cell Res. 113, 445-450.

NACHMANSON, D. (1959) in Chemical and Molecular Basis of Nerve Activity, Academic Press, New York.

NASTUK, W.C., STRAUSS, A.J.L. and OSSERMAN, K.E. (1959) Am. J. Med. 26, 394-409.

NASTUK, W.L., PLESCIA, O.J. and OSSERMAN, K.E. (1960) Proc. Soc. Exp. Biol. Med. 105, 177-184.

NATHANSON, N.M. and HALL, Z.W. (1979) Biochem. 18, 3392-3401.

NATVIG, J.B. and KUNKEL, H.G. (1973) Adv. Immunol. 16, 1-54.

NEHER, E. and SAKMANN, B. (1976) J. Physiol. 258, 705-729.

NEUMANN, D., FRIDKIN, M. and FUCHS, S. (1984) Biochem. Biophys. Res. Commun. 121, 673-679.

NEWSOM-DAVIS, J. and VINCENT, A. (1982) Immunol. Today 3, 149-151.

NEWSOM-DAVIS, J., WILLCOX, N., SCADDING, G., CALDER, L. and VINCENT, A. (1981) Ann. N.Y. Acad. Sci. 377, 393-401.

NODA, M., TAKAHASHI, H., TANABE, T., TOYOSATO, M., FURUTANI, Y., HIROSE, T., ASAI, M., INAYAMA, S., MIYATA, T. and NUMA, S. (1982) Nature 299, 793-797.

NODA, M., TAKAHASHI, H., TANABE, T., TOYOSATO, M., KIKYOTANI, S., HIROSE, T., ASAI, M., TAKASHIMA, H., INAYAMAS, S., MIYATA, T. and NUMA, S. (1983a) Nature 301, 251-255.

NODA, M., TAKAHASHI, H., TANABE, T., TOYOSATO, S., KIKYOTANI, S., FURUTANI, Y., HIROSE, T., TAKASHIMA, H., INAYAMA, S., MIYATA, T. and NUMA, S. (1983b) Nature 302, 528-532.

NODA, M., FURUTANI, Y., TAKAHASHI, H., TOYOSATO, M., TANABE, T., SHIMIZU, S., KIKYOTANI, S., KAYANO, T., HIROSE, T., INAYAMA, S. and NUMA, S. (1983c) Nature 305, 818-823.

O'BRIEN, R.D., ELDEFRAWI, M.E. and ELDEFRAWI, A.T. (1972) Ann. Rev. Pharmacol. 12, 19.

OCHOA, E.L.M., DALZIEL, A.W. and McNAMEE, M.G. (1983) Biochim.

Biophys. Acta 727, 151-162.

OH, T.H. and MARKELONIS, G.J. (1980) Proc. Natl. Acad. Sci USA 77, 6922-6925.

OLD, L.J., STOCKERT, E., BOYSE, E.A. and KIM, J.H. (1968) J. Exp. Med. 127, 523-539.

OLSEN, R.W., MEUNIER, J.C. and CHANGEUX, J.P. (1972) FEBS Lett. 28, 96-100.

O'NEILL, M.C. and STOCKDALE, F.E. (1972) J. Cell Biol. 52, 52-65.

OOSTERHUIS, H.J.G.H., BETHLEM, J. and FELTKAMP, T.E.W. (1968) J. Neurol. Neurosurg. Psychiatry 31, 460.

OSSERMAN, K.E. and GENKINS, G. (1971) Mt. Sinai J. Med. 38, 497.

PAPATESTAS, A.E., ALPERT, L.I., OSSERMAN, K.E. and KARK, A.E. (1971) Am. J. Med. 50, 465-474.

PARKES, J.D. and McKINNA, J.A. (1966) Lancet i, 388-391.

PATERSON, B. and PRIVES, J. (1973) J. Cell. Biol. 59, 241-245.

PATRICK, J. and LINDSTROM, J. (1973) Science 180, 871-872.

PATRICK, J. and BERMAN, P.W. (1980) in The Cell Surface and Neuronal Function (COTMAN, C.W., POSTE, G. and NICOLSON, G.L. eds.) pp. 157-190, Elsevier/North Holland Biomedical Press.

PATRICK, J., HEINEMANN, F., LINDSTROM, J., SCHUBERT, D. and STEINBACH, J.H. (1972) Proc. Natl. Acad. Sci USA 69, 2762-2766.

PATRICK, J., LINDSTROM, J., CULP, B. and McMILLAN, J. (1973) Proc. Natl. Acad. Sci. USA 70, 3334-3338.

PENN, A., CHANG, H., LOVELACE, R., NIEMI, W. and MIRANDA, A. (1976) Ann. N.Y. Acad. Sci. 274, 354-376.

PHILLIPS, H.J. (1973) in Tissue Culture: Methods and Applications (KRUSE, P.F. and PATTERSON, M.K. eds.) pp.406-408, Academic Press, New York and London.

POGOGEFF, I.A. and MURRAY, M.R. (1946) Anat. Rec. 95, 321-335.

PRESSMAN, D. (1970) Immunochem. 7, 870.

PRIVES, J.M. and OLDEN, K. (1980) Proc. Natl. Acad. Sci. USA 77, 5263-5267.

PRIVES, J., SILMAN, I. and AMSTERDAM, A. (1976) Cell 7, 543-550.

PRIVES, J., HOFFMAN, L., TARRAB-HAZDAI, R., FUCHS, S. and AMSTERDAM, A. (1979) Life Sci. 24, 1713-1718.

PUMPLIN, D.W. and FAMBROUGH, D.M. (1982) Ann. Rev. Physiol. 44, 319-335.

RAFTERY, M.A., SCHMIDT, J., CLARK, D.G. and WOLCOTT, R.G. (1971) Biochem. Biophys. Res. Commun. 45, 1622-1629.

RAFTERY, M., VANDLEN, R., REED, K. and LEE, T. (1976) Cold Spring Harbor Symp. Quant. Biol. 40, 193-202.

RAFTERY, M.A., BLANCHARD, S., ELLIOT, J., HARTIG, P., MOORE, H.P., QUASTEL, U., SCHMERLIK, M., WITZEMANN, V. and WU, W. (1979) in Advances in Cytopharmacology (CECCARELLI, B. and CLEMENTI, F. eds.) Vol.3, pp.159-189, Raven Press, New York.

RASH, J.E., ALBUQUERQUE, E.X., HUDSON, C.S., MAYER, R.F. and SATTERFIELD, J.R. (1976) Proc. Natl. Acad. Sci. USA 73, 4584-4588.

RASH, J.E., HUDSON, C.S., GRAHAM, W.F., MAYER, R.F., WARNICK, J.E. and ALBUQUERQUE, E.X. (1981) Lab. Invest. 44, 519-530.

REBOUCHE, C.J. (1977) Biochim. Biophys. Acta 471, 145-155.

REINHARD, R., WALTERS, D. and MAELICKE, A. (1981) Eur. J. Biochem. 119, 215-224.

RESKE-NIELSON, E., DALBY, A. and DALBY, M. (1965) Acta Neurologica Scandinavica 41 Suppl. 13, 289-296.

RINALDINI, L.M. (1959) Exp. Cell Res. 16, 477-505.

ROSS, M.J., KLYMKOWSKY, M.W., AGARD, D.A. and STROUD, R.M. (1977) J. Mol. Biol. 116, 635-659.

RUSSELL, D (1953) J. Path. Bact. 65, 279-289.

SAFWENBERG, J.L., HAMMARSTROM, L., LINDBLOM, J.B., MATELL, G., MOLLER, E., OSTERMANN, P.O. and SMITH, S.I.E. (1978) Tissue Antigens 12, 136-142.

SAHASHI, K., ENGEL, A.G., LINDSTROM, J., LAMBERT, E.H. and LENNON, V. (1978) J. Neuropathol. Exp. Neurol. 37, 212-223.

SAITOH, T., WENNOGLE, L. and CHANGEUX, J.P. (1979) FEBS Lett. 108, 489-494.

SAITOH, T., OSWALD, R., WENNOGLE, L.P. and CHANGEUX, J.P. (1980) FEBS Lett. 116, 30-36.

SANTA, T., ENGEL, A.G. and LAMBERT, E.H. (1972) Neurology 22, 71-82.

SARGENT, P.B., HEDGES, B.E., TSAVALER, L., CLEMMONS, L., TZARTOS, S. and LINDSTROM, J.M. (1984) J.Cell Biol. 98, 609-618.

SCHMIDT, J. (1984) J. Biol. Chem. 259, 14033-14037.

SCHMIDT, J. and RAFTERY, M.A. (1973) Analyt. Biochem. 52, 349-354.

SCHUBERT, D., TARIKAS, H., HUMPHREYS, S., HEINEMANN, S. and PATRICK, J. (1973) Dev. Biol. 33, 18-37.

SCHUETZE, S.M., FRANK, E.F. and FISCHBACH, G.D. (1978) Proc.Natl. Acad. Sci USA 75, 520-523.

SEYBOLD, M.E. (1983) JAMA 250, 2516-2521.

SHERMAN, L.A., HARWIG, S. and HAYNE, O.A. (1974) Int. J. Appl. Radiation Isotopes 25, 81-85.

SHORR, R.G., DOLLY, J.O. and BARNARD, E.A. (1978) Nature 274, 283-284

SHORR, R.G., LYDDIATT, A., LO, M.M.S., DOLLY, J.O. and BARNARD, E.A. (1981) Eur. J. Biochem. 116, 143-153.

SILMAN, I. and KARLIN, A. (1967) Proc. Natl. Acad. Sci. USA 58, 1664-1668.

SIMPSON, J.A. (1958) Brain 81, 112-144.

SIMPSON, J.A. (1960) Scott. Med. J. 5, 419-436.

SIMPSON, J.A. (1974) in Disorders of Voluntary Muscle (WALTON, J.N.

ed.) pp.653-692, Churchill Livingstone, London.

SIMPSON, J.A. (1978a) Muscle and Nerve 1, 45-56.

SIMPSON, J.A. (1978b) Muscle and Nerve 1, 151-156.

SKRBIC, T.R., YASIN, R., van BEERS, G., BULIEN, D. and THOMPSON, E.J. (1975) Biochem. Soc. Trans. 3, 496-497.

SOBEL, A., HEIDMANN, T., HOFLER, J. and CHANGEUX, J.P. (1978) Proc. Natl. Acad. Sci. USA 75, 510-514.

SOKOLOVSKY, M., RIORDAN, J.F. and VALLEE, B.L. (1966) Biochem. 5, 3582-3589.

SOUROUJON, M.C., MOCHLY-ROSEN, D., GORDON, A.S. and FUCHS, S. (1983) Muscle and Nerve 6, 303-311.

STEPHENSON, F.A., HARRISON, R. and LUNT, G.G. (1981) Eur. J. Biochem. 115, 91-97.

STEVENSON, C.T. and DORRINGTON, K.J. (1970) Biochem. J. 118, 703-712.

STRAUSS, A.J.L., SEEGAL, B.C., HSU, K.C., BURKHOLDER, P.M., NASTUK, W.L. and OSSERMAN, K.E. (1960) Proc. Soc. Exp. Biol. Med. 105, 184-191.

SUGIYAMA, H., BENDA, P., MEUNIER, J. and CHANGEUX, J.P. (1973) FEBS Lett. 35, 124-128.

SUMIKAWA, K., HOUGHTON, M., SMITH, J.C., BELL, L., RICHARDS, B.M. and BARNARD, E.A. (1982) Nucleic Acids Res. 10, 5809-5822.

SYTKOWSKI, A.J., VOGEL, Z. and NIRENBERG, M. W. (1973) Proc. Natl. Acad. Sci. USA 70, 270-274.

TARRAB-HAZDAI, R., GEIGER, B., FUCHS, S. and AMSTERDAM, A. (1978) Proc. Natl. Acad. Sci. USA 75, 2497-2501.

TARRAB-HAZDAI, R., SCHMIDT-SOLE, Y., MOCHLY-ROSEN, D. and FUCHS, S. (1980) FEBS Lett. 118, 35-38.

TENG, N.N.H. and FISZMAN, M.Y. (1976) J. Supramol. Struct. 4,

381-387.

TEPPERMAN, K., MORRIS, G., ESSIEN, F. and HEYWOOD, S.M. (1975) J. Cell Physiol. 86, 561-566.

TINDALL, R.S.A. (1981) Ann. N.Y. Acad. Sci. 377, 316-331.

TOYKA, K.V., DRACHMAN, D.B., PESTRONK, A. and KAO, I. (1975) Science 190, 397-399.

TURNBULL, G.M., HARRISON, R. and LUNT, G.G. (1985) Int. J. Dev. Neurosci. 3, 123.

TZARTOS, S.J. and LINDSTROM, J.M. (1980) Proc. Natl. Acad. Sci. USA 77, 755-759.

TZARTOS, S. and LINDSTROM, J. (1981) in Monoclonal Antibodies in Endocrine Research (FELLOWS, R. and EISANBARTH, G. eds.) pp.69-86, Raven Press.

TZARTOS, S.J., RAND, D.E., EINARSON, B.L. and LINDSTROM, J.M. (1981) J. Biol. Chem. 256, 8635-8645.

TZARTOS, S., SEYBOLD, M.E. and LINDSTROM, J. (1982) Proc. Natl. Acad. Sci. USA 79, 188-192.

UENO, S., KANG, J., TAKEUCHI, H., TAKAHASHI, M. and TARUI, S. (1980) Clin. Exp. Immunol. 41, 13-18.

URBANIAK, S.J., PENHALE, W.J. and IRVINE, W.J. (1973) Clin. Exp. Immunol. 15, 345-354.

VALDERRAMA, R., WEILL, C.L., McNAMEE, M.G. and KARLIN, A. (1976) Ann. N.Y. Acad. Sci. 274, 108-115.

VAN der GELD, H., FELTKAMP, T.E.W., van LOGHEM, J.J., OOSTERHUIS, H.J.G.H. and BIEMOND, A. (1963) Lancet ii, 373-375.

VANDLEN, R., WILSON, C., EISENBACH, J. and RAFTERY, M. (1979) Biochem. 18, 1845-1854.

VINCENT, A. (1980) Physiol. Rev. 60, 756-824.

VINCENT, A. and BILKHUS, M. (1982) J. Immunol. Methods 51, 359-369.

VINCENT, A. and NEWSOM-DAVIS, J. (1979) in Advances in Cytopharmacology (CECCARELLI, B. and CLEMENTI, F. eds.) Vol.3, pp.269-279, Raven Press, New York.

VINCENT, A. and NEWSOM-DAVIS, J. (1982) Clin. Exp. Immunol. 49, 257-265.

VINCENT, A., NEWSOM-DAVIS, J. and MARTIN, V. (1978) Lancet i, 1254.

VOGEL, Z., SYTKOWSKI, A.J. and NIRENBERG, M.W. (1972) Proc. Natl. Acad. Sci. USA 69, 3180-3184.

WALDMANN, T.A., BLAESE, R.M., BRODER, S. and KRAKAUER, R.S. (1978) Ann. Int. Med. 88, 226-238.

WALKER, M.B. (1934) Lancet 226, 1200-1201.

WALLIS, I., KOENIG, E. and ROSE, S. (1980) Biochim. Biophys. Acta 599, 505-517.

WATKINS, W.M. (1966) Methods in Enzymol. 8, 700-711.

WEBER, K. and OSBORN, M. (1969) J. Biol. Chem. 244, 4406-4112.

WEINBERG, C.B. and HALL, Z.W. (1979) Proc. Natl. Acad. Sci. USA 76, 504-508.

WENNOGLE, L.P. and CHANGEUX, J.P. (1980) Eur. J. Biochem. 106, 381-393.

WENNOGLE, L.P., OSWALD, R.E., SAITOH, T. and CHANGEUX, J.P. (1981) Biochem. 20, 2492-2497.

WERMAN, R. (1966) Comp. Biochem. Physiol. 18, 745.

WERNECK, L.C. (1982) Arq. Neuro-Psiquiatr. 40, 67-76.

WESTWOOD, J.H., THOMAS, P., EDWARDS, R.G. and MUNRO NEVILLE, A. (1975) Protides Biol. Fluids Proc. Colloq. Bruges 24, 413-418.

WHITING, P.J., VINCENT, A. and NEWSOM-DAVIS (1983) J. Neuroimmunol. 5, 1-9.

WILLMER, E.N. (1965) in Cells and Tissues in Culture, Vol.1 (WILLMER, E.N. ed.) pp.143-176, Academic Press, New York.

WILSON, A. and STONER, H.B. (1944) Quart. J. Med. 13, 1-18.

WISE, D.S., WALL, J. and KARLIN, A. (1981) J. Biol. Chem. 256, 12624-12627.

WITKOWSKI, J.A. (1977) Biol. Rev. 52, 431-476.

WITKOWSKI, J.A., DURBRIDGE, M. and DUBOWITZ, V. (1976) In vitro 12, 98-106.

WONNACOTT, S., HARRISON, R., LUNT, G.G. and BARKAS, T. (1980a) Eur. J. Biochem. 108, 621-629.

WONNACOTT, S., HARRISON, R. and LUNT, G.G. (1980b) Life Sci. 27, 1769-1775.

YAFFE, D. (1968) Proc. Natl. Acad. Sci. USA 61, 477-483.

YASIN, R., van BEERS, G., NURSE, K.C.E., AL-ANI, S., LONDON, D.N. and THOMPSON, E.J. (1977) J. Neurol. Sci. 32, 347-360.

YASUKO, H., ICHIRO, K. and OZAWA, E. (1981) Dev. Growth Differ. 23, 249-254.

YOSHIDA, T., TSUCHIYA, M., ONO, A., YOSHIMATSU, H., SATOYOSHI, E. and TSUJI, K. (1977) J. Neurol. Sci. 32, 195-201.

YUNIS, E.J., STUTMAN, O. and GOOD, R.A. (1971) Ann. N.Y. Acad. Sci. 183, 205-220.

ZACKS, S.I., BAUER, W.C. and BLUMBERG, J.J. (1961) Nature 190, 280-281.

ZINGSHEIM, H.P., BARRANTES, F.J., FRANK, J., HANICKE, W. and NEUGEBAUER, D.C. (1982) Nature 299, 81-83.

ZURN, A.D. and FULPIUS, B.W. (1977) Eur. J. Immunol. 8, 529-532.